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THE EFFECT OF CHROMATIN COMPACTION MODIFICATION ON THE EFFICACY OF ANTICANCER MEDICAMENTS

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더는아프지않게
네가웃을수있게”
Promise (2018), *JIMIN*, *RM*

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RESUMEN

INTRODUCCIÓN

Cáncer y su tratamiento

El cáncer es la segunda causa de muerte en todo el mundo. Consiste en un grupo de enfermedades que se caracterizan por la proliferación incontrolada de células y la capacidad de dichas células para invadir otras partes del cuerpo. La transformación de una célula normal en una célula tumoral es el resultado de múltiples mutaciones genéticas y modificaciones epigenéticas que provocan cambios importantes en la biología celular. Hanahan y Weinberg describen 6 cambios en la biología celular como características del desarrollo del tumor: capacidad para mantener la proliferación crónica, evasión de supresores del crecimiento, resistencia a la muerte celular, inmortalidad replicativa mantenida, inducción de angiogénesis y activación de invasión y metástasis.

Los tratamientos contra el cáncer comprenden principalmente la cirugía, la quimioterapia citotóxica, la terapia dirigida, la radioterapia, la terapia endocrina y la inmunoterapia. La quimioterapia es uno de los métodos prevalentes utilizados para tratar tumores malignos. Incluye más de 100 tipos de agentes cuyo mecanismo generalmente se basa en la alteración del ciclo celular (de manera dependiente o independiente de la fase del ciclo celular) que produce la muerte celular. La quimioterapia convencional incluye antimetabolitos, agentes alquilantes y derivados de platino, inhibidores y venenos de topoisomerasa, antibióticos antitumorales y agentes antimicrotubulares.

La quimioterapia se enfrenta tres obstáculos principales que limitan su eficacia: cantidad insuficiente de medicamento que alcanza la diana, resistencia a los medicamentos contra el cáncer y toxicidad de los medicamentos debido su acción en células normales.

En primer lugar, un medicamento que no alcanza un tumor no será efectivo. Cualquier medicamento debe estar disponible en su lugar de acción en una concentración suficiente. Cuando se administra por vía oral, el fármaco debe absorberse en la sangre por lo que la absorción intestinal deficiente constituiría la primera limitación. En segundo lugar, tanto en tratamientos orales como intravenosos, los agentes quimioterapéuticos pueden metabolizarse o excretarse. Después de llegar al área del tumor, los medicamentos deben cruzar la pared del vaso y penetrar en el intersticio. Un exceso de colágeno en la composición de la matriz extracelular del tumor puede bloquear la difusión de fármacos hacia el tumor. Además, la alteración de la vasculatura tumoral limitará la penetración del fármaco. Asimismo, la hipoxia y la falta de nutrientes en las células tumorales lejos de los vasos sanguíneos mantienen estas células en un estado inactivo, menos receptivas a muchos medicamentos contra el cáncer que requieren células metabólica o proliferantemente activas. La hipoxia obliga a las células a emplear la glucólisis anaeróbica para generar energía. Esto desencadenará una acidificación del intersticio tumoral. En condiciones ácidas, los medicamentos básicos (como la doxorrubicina) se pueden protonar, lo que podría interferir con su acción en las células. Otro desafío importante para la quimioterapia es la barrera hematoencefálica (BBB), que impide la entrada al cerebro de la mayoría de los medicamentos anticancerosos convencionales.

La resistencia a los medicamentos sigue siendo un problema importante en las terapias contra el cáncer y es responsable de la mayoría de las recaídas, una de las principales causas de muerte en pacientes con cáncer. La resistencia a la quimioterapia se puede dividir en dos grandes categorías, intrínseca o adquirida, según la respuesta inicial del tumor a la terapia. La resistencia adquirida: cuando el tumor se reduce inicialmente en respuesta al tratamiento pero, durante el proceso de tratamiento, se vuelve resistente y comienza a crecer nuevamente; y resistencia intrínseca, cuando el tumor es resistente antes de ser tratado. Existen varios mecanismos y factores que conducen a la resistencia: 1) transporte alterado del fármaco (absorción, exportación y secuestro), 2) metabolización del fármaco (activación, inactivación), 3) alteración de la diana, 4)

respuesta al daño del ADN, 5) cambios epigenéticos, 6) inhibición de la muerte celular, 7) efecto del microambiente tumoral, 8) presencia de heterogeneidad tumoral y CSC (células madre tumorales). La resistencia puede limitarse a los medicamentos a los que están expuestos los pacientes (resistencia a un solo agente) o ser un mecanismo simultáneo de ausencia de respuesta a múltiples medicamentos con diferentes estructuras y mecanismos de acción. [22-24]

Accesibilidad de cromatina y ADN

La quimioterapia convencional sigue siendo la primera línea de tratamiento contra el cáncer, y los medicamentos dirigidos al ADN desempeñan un papel importante en la terapia contra el cáncer. Su tasa de éxito está lejos de ser óptima, por lo tanto, es esencial desarrollar nuevas estrategias y enfoques para mejorar la supervivencia del paciente. Los fármacos que actúan uniéndose al ADN se enfrentan dificultades una vez dentro de la célula para unirse al ADN. Por ejemplo, se sabe que en las células tratadas con CDDP (cisplatino) solo el 1 % del CDDP dentro de la célula se une al ADN nuclear. Nos centraremos en el estudio de este problema utilizando dos medicamentos de diferentes familias como modelos: CDDP y DOX (doxorrubicina).

El CDDP es un tratamiento ampliamente utilizado cuyo mecanismo de acción de CDDP se ha asociado a su capacidad de formar aductos con las bases de purina del ADN, interfiriendo con los mecanismos de reparación del ADN, inhibiendo la síntesis de ADN y la mitosis, provocando daños en el ADN e induciendo la apoptosis en las células cancerosas. Desafortunadamente, muchos tumores desarrollan resistencia al tratamiento con CDDP. La combinación con otros fármacos es una buena manera de superar la resistencia a los medicamentos y reducir su toxicidad. La DOX se intercala en el ADN y forma aductos. Esta unión evita la actividad de las polimerasas de ADN y ARN, lo que bloquea la síntesis de ADN y ARN y desencadena la apoptosis.

El ADN genómico de las células eucariotas está empaquetado en un complejo nuclear asociado con proteínas y ARN llamado

cromatina. La unidad fundamental de la cromatina son los nucleosomas, en los que 147 pares de bases de ADN se envuelven alrededor de un octámero de cuatro histonas centrales (H2A, H2B, H3 y H4). El ADN conector entre nucleosomas está unido a la histona H1 para compactar la cromatina. El ADN se une de manera estable con las histonas mediante la interacción de los fosfatos cargados negativamente con las cargas positivas de las histonas (proteínas básicas). La cromatina se enrolla heterogéneamente y compacta esta estructura básica en estados de orden superior. Al limitar la accesibilidad del ADN a la maquinaria nuclear la cromatina regula procesos como la transcripción génica, la replicación o la reparación del ADN.. Por lo tanto, la cromatina debe ser dinámica permitiendo cambios locales en su estructura. Este comportamiento dinámico se logra mediante la existencia de variantes de histonas y modificaciones postraduccionales (PTM). Las variantes de histonas muestran diferentes niveles de afinidad con el ADN, sitios específicos para PTM o para ser reconocidos por enzimas como chaperonas de histona, remodeladores de cromatina y enzimas modificadoras de histona y ADN. Las PTM como acetilación, metilación, fosforilación, ubiquitinación, modifican residuos específicos de histonas y otras proteínas. Pueden modificar histonas libres o histonas dentro de un nucleosoma ensamblado. Las PTM pueden modificar las interacciones histona-histona, las interacciones ADN-histona y la interacción de la cromatina con otros complejos y factores. De todas las modificaciones conocidas, la acetilación tiene el mayor potencial para desplegar la cromatina, ya que neutraliza la carga básica de la lisina. El ensamblaje, desensamblaje y distribución de histonas y nucleosomas está controlado por dos grandes familias de proteínas: chaperonas de histonas y complejos de remodelación de nucleosomas. Las histonas se sintetizan en el citoplasma y se unen inmediatamente por chaperonas de histonas (necesarias para el transporte, la formación adecuada y específica de nucleosomas, la transferencia y el control de la degradación de histonas). Después de que se formen los nucleosomas, los remodeladores de cromatina pueden cambiar su composición de variantes de histonas, el nivel de compactación y la posición en relación con motivos de la secuencia.

Del mismo modo que la cromatina actúa como una barrera natural en condiciones fisiológicas para la maquinaria nuclear, queremos explorar si tiene un efecto similar en la unión de fármacos al ADN. Resultados previos obtenidos en nuestro grupo muestran que Ag_3-AQC (Clusters atómicos cuánticos de tres átomos de plata) modifican la compactación de la cromatina y, en consecuencia, mejoran la eficacia de fármacos como oxaliplatino, carboplatino, gemcitabina, CDDP y DOX en células tumorales in vitro y de CDDP en modelos de ratón. Dado que la cromatina podría actuar como una barrera para los fármacos que se unen al ADN, exploramos si el mecanismo que se encuentra en los AQC es específico o si una reducción en la compactación de la cromatina puede mejorar la accesibilidad del ADN a los fármacos y, por lo tanto, la eficacia de los agentes quimioterapéuticos. Para probar esta hipótesis, cuantificamos la unión de CDDP y DOX al ADN en varios modelos de compactación de cromatina reducida.

Primero, en colaboración con StemCells& Human DiseasesLab, comparamos la unión de CDDP y DOX al ADN en dos ESC murinos (células madre embrionarias) de la línea parental cj7. Estas dos líneas se diferencian por una mutación que tiene como resultado un fenotipo con menor de compactación de cromatina. Posteriormente, para estudiar el efecto en un modelo más controlado, analizamos el efecto de una reducción parcial de la histona H4 en un modelo de levadura en colaboración con DNA Repair y Genome Integrity Group. Como la disponibilidad de histonas ha demostrado afectar la compactación de la cromatina tanto en levaduras como en humanos. Comparamos dos cepas de Saccharomyces cerevisiae, una con expresión normal de H4 y la otra en la que la expresión de histona H4 fue impulsada por un promotor tet regulado. En esta cepa, la expresión de H4 es inducida por el tratamiento con doxiciclina, que nunca alcanza niveles de expresión normales. Sin embargo, existen muchas diferencias en la respuesta de la levadura a la modificación del ensamblaje de nucleosomas en comparación con los humanos; y queríamos estudiar un modelo con potencial aplicación clínica. Se seleccionaron proteínas relacionadas con la formación de histonas y de nucleosomas: CASP8AP2 y CAF-1. La proteína 2 asociada a caspasa 8

(CASP8AP2) es un regulador transcripcional de genes de histonas y el factor de ensamblaje de cromatina 1 (CAF-1) es una chaperona de histonas de tres subunidades (con las subunidades p150, p60 y p48) que deposita específicamente las histonas H3-H4 recién sintetizadas en el ADN que se está replicando durante la fase S. Para reducir la compactación de la cromatina, la línea celular humana de adenocarcinoma A549 se transfectó con siARN dirigido al ARNm de CASP8AP2 y al ARNm de chaf1b (que codifica la subunidad p60 de CAF-1). Los siARN son ARN de doble cadena de pequeño tamaño (en este caso, 19 pares de bases) que interfieren con la expresión mediante la unión al ARNm y su posterior escisión. [86-89]Por último, estudiamos el efecto de algunos medicamentos ampliamente estudiados en uso clínico en el tratamiento del cáncer: los inhibidores deacetilasas de histonas (iHDAC). Los iHDAC inhiben deacetilasas de histonas (HDAC), una de las principales clases de enzimas que realizan PTM. Las HDACs catalizan la hidrólisis de los residuos de N-acetil lisina en las histonas y actúan en oposición a la acción de las acetil-transferasas de histonas. La hiperacetilación tiene como resultado una cromatina más relajada (menos compacta). Las HDAC se dividen en cuatro clases principales de enzimas dependientes de zinc Clase I (HDAC 1, 2, 3, 8), Clase IIa (HDAC 4, 5, 7, 9), Clase IIb (HDAC 6, 10) y Clase IV (HDAC 11) y las enzimas Clase III no dependientes de zinc (sirtuinas dependientes de NAD +). Seleccionamos tres iHDAC: tricostatina A (TSA), suberanilohidroxámico (SAHA; vorinostat; Zolinza®) y valproato (VPA). TSA y SAHA son hidroximatos que inhiben todos las HDAC. El VPA es un ácido graso de cadena corta que solo inhibe los HDAC de clase I y IIa. Como en modelos anteriores, estudiamos la unión de CDDP y DOX al ADN en la línea celular A549.

OBJETIVO

Estudiar el efecto de la compactación de la cromatina y sus posibles modificaciones farmacológicas para aumentar la actividad terapéutica de los fármacos que se unen al ADN.

MATERIALES Y MÉTODOS

El CDDP se preparó en PBS a 1 mg / ml y la DOX se suspendió en DMSO (sulfóxido de dimetilo) a 1 mg / ml también. La unión de CDDP al ADN se cuantificó directamente como una relación de contenido de Pt / contenido de fósforo en muestras de ADN de células tratadas con CDDP. La cantidad de platino y fósforo se determinó por espectrometría de masas utilizando un ICP-MS BRUCKER 820-MS con un nebulizador de vidrio de bajo flujo Micromist y una cámara de pulverización de doble paso con enfriamiento Peltier (3 ° C) y antorcha de cuarzo (BrukerCorp). La ICP-MS fue realizada por Verónica Piñeiro de la Unidade de Análise Elemental (CACTUS, Lugo). La unión de DOX se midió usando su propia fluorescencia de dos maneras: cuantificando la fluorescencia de DOX en las células mediante citometría de flujo usando citómetros de flujo GuavaEasyCyte o BD Accuri ™ C6 Plus y cuantificando la fluorescencia nuclear de DOX mediante microscopía confocal realizada con el microscopio confocal Leica TCS SP8 (las imágenes fueron obtenidos por Marta Picado Barreiro del Servicio Confocal de IDIS, Santiago de Compostela). Las proyecciones máximas se obtuvieron utilizando el software Leica LAS X, y luego la fluorescencia nuclear se cuantificó con el software ImageJ (RawIndent).

Todos los experimentos con el modelo ESC se realizaron en colaboración con Yara Souto Becerra del grupo de Células Madre y Enfermedades Humanas (CiMUS, Santiago de Compostela) bajo la supervisión de Miguel Fidalgo Pérez. El modelo ESC consiste en dos líneas de células madre embrionarias de ratón de la línea parental CJ7. Uno es de tipo salvaje (WT) y un mutante para Zmym2 (KO) obtenido después de la inserción por GeneTrap de un gen para la resistencia a los antibióticos G418 en el gen Zmym2, que da como resultado una proteína no funcional. La línea KO fue creada por el Dr. Jianlong Wang Lab (Icahn Schoolof Medicine, Mount Sinai Hospital, NY).

Todos los experimentos con modelos de levadura se realizaron en colaboración con Tomás Lama Díaz del Laboratorio de Reparación de

ADN e Integridad del Genoma (CiMUS, Santiago de Composela), bajo la supervisión de Miguel González Blanco. En el modelo de levadura se usaron dos cepas. Una cepa con H4reducia: ΔH4; y una cepa con expresión normal de H4, WT. La cepa parcialmente agotada de H4, ΔH4, fue creada por Prado y Aguilera (Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Sevilla, España) [85] y es un mutante nulo para las dos copias del gen de la histona H4 (hhf1Δ hhf2Δ) en la cepa BY4741 y contiene un plásmido (p413TARtetH4) que expresa HHF2 bajo el control del promotor bacteriano tet. Este plásmido alberga copias de las fusiones tetR'-SSN6 y tetR-VP16, que regulan positivamente el promotor tet en respuesta a la doxiciclina de una manera dependiente de la dosis (utilizado en 5 μg / mL o 0.25 μg / mL). La otra cepa, WT, es una cepa con el mismo fondo que ΔH4, que se transformó con el plásmido pRS413 para ofrecer la capacidad de crecer sin suplementación con histidina (presente en la otra cepa). Para verificar que nuestros cultivos contengan cepas de interés, los niveles de H4 se estudiaron mediante dos métodos: Western Blot e inmunotinción de H4 en células seguida por la cuantificación por microscopía de fluorescencia.

Los modelos de silenciamiento e iHDAC se realizaron con la línea celular de adenocarcinoma de pulmón humano (A549) obtenida de DMSZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Alemania). En los modelos de silenciamiento, la línea celular de adenocarcinoma de pulmón humano A549 se silenció usando ARNsi de Dharmacon y el reactivo de transfección DharmaFECT. Para estudiar la expresión después del silenciamiento se realizó RT-qPCR (reverso transcripción seguida de *Real Time PCR quantification).*

En cuanto a los iHDACs, se suspendió TSA en DMSO en una concentración de 50 μM, SAHA en DMSO, 9,4 mM; El VPA y el clorhidrato de procaína se prepararon en H2Omq estéril a 20 M y 10 M, respectivamente. El efecto en la viabilidad celular de la combinación de iHDAC (en dosis subletales) y CDDP se estudió utilizando ensayos MTT o Guava Via Count (Millipore).

El ciclo celular de las células se estudió mediante el uso de STYOX Green (levaduras) y yoduro de propidio (A549) y se midió por citometría de flujo.

Todos los análisis estadísticos se realizaron con el software Graph Pad Prism Versión 13 (GraphPad Software, Inc., La Jolla, EE. UU.). Las diferencias se consideraron significativas para $p < 0,05$. Los resultados se expresan como media ± desviación estándar. Las medias se compararon usando una t en espiral de análisis de Student en dos muestras de comparación cuando las muestras presentan distribución gaussiana y ambas poblaciones tienen la misma varianza. Cuando se compara la media de más de un grupo con la distribución gaussiana y las poblaciones homocedásticas, se realizó un ANOVA unidireccional seguido de análisis de comparación múltiple de Dunnet. Se usaron dos pruebas de normalidad para probar la distribución gaussiana D'Agostino-Pearson (cuando las muestras eran grandes) y Shapiro-wilk (para muestras pequeñas) y la prueba Browne-Forsythe se usó para probar igualmente las variaciones. Cuando se rechazó la normalidad, se realizó el ensayo de Mann-Whitney. Cuando las poblaciones eran heterocedásticas, se seleccionó la corrección de Welch de t-Student y ANOVA (seguido de Dunnet 3). En muestras con $N > 50$ se realizó un análisis paramétrico incluso cuando se rechazó la normalidad en base al teorema del límite central.

RESULTADOS Y DISCUSIÓN

En el modelo de células embrionarias de ratón, tanto el CDDP como la DOX se unieron más al ADN (entre un 10 -30% más) en las células mutantes, con menor compactación de la cromatina. Este modelo es interesante como primer enfoque ya que no requiere ningún tipo de modificación farmacológica y que la reducción de la compactación de la cromatina no tiene efectos tóxicos permitiendo el mantenimiento de estas líneas. Además, la proteína no funcional en el mutante Zmym2 se ha descrito como una subunidad de un complejo que incluye las HDACs 1 y 2. Por lo que el cambio en la compactación de la cromatina podría deberse a un aumento en la acetilación de histonas. Sin embargo, otros factores podrían estar

alterando la unión de CDDP y DOX al ADN, por ello, la siguiente aproximación se realizó con un modelo genético de levaduras, en que la reducción de la expresión de H4 tiene como consecuencia una menor compactación de la cromatina. Igual que en las células embrionarias, se observó un aumento de la unión de estos fármacos al ADN en la cepa de levadura con expresión parcial de H4. El aumento observado fue del 400% y 300% en promedio en el caso de CDDP y DOX, respectivamente.

Para poder comprobar nuestra hipótesis en un modelo más cercano a la aplicación clínica, se realizó una aproximación similar a la usada en levaduras: la modificación genética de la expresión de histonas, y además de un factor fundamental en la deposición de histonas para la formación de nuevos nucleosomas durante la replicación. Se realizó mediante el silenciamiento de CAF-1 (chaperona de histonas, implicada en la formación de nucleosomas de novo) y CASP8AP2 (factor de transcripción de histonas canónicas) en células de la línea humana A549 de adenocarcioma de pulmón. El silenciamiento fue comprobado mediante RTq-PCR utilizando un ensayo Taq-Man®. Debido a la dificultad que presentaban estos modelos tanto por la toxicidad inherente al silenciamiento de CAF-1 y CASP8AP, que son necesarias durante la replicación del ADN, como de la dificultad asociada al uso de silenciamiento en clínica, sólo se estudió la unión de la DOX es estas células. El silenciamiento de CAF-1 y de CASP8AP2 produjo un aumento en la unión de DOX al ADN (10% en las células con silenciamiento de CASP8AP2 y 50% , en CAF-1). Los resultados de este modelo concuerdan con los obtenidos en las aproximaciones previas de este trabajo, y también con los resultados obtenidos en este laboratorio previamente en los que el tratamiento con Ag3-AQCs producía una disminución de la compactación de la cromatina durante la fase S, que provocaba un aumento en la unión de fármacos al ADN (de hasta un 500%).

La compactación de la cromatina también cambia durante la fase G1 del ciclo celular, mediante la acción de remodeladores de la cromatina. Estos complejos incluyen entre otros a las deacetilasas de histonas. La acetilación de las histonas reduce su afinidad por el ADN, por ello el uso de inhibidores de deacetilasas de histonas, provoca la

descompactación de la cromatina. Este modelo tiene algunas ventajas en comparación con los anteriores, es barato, sencillo y tiene un impacto directo en la clínica. Dado que la acetilación puede ocurrir en cualquier momento desde la formación de histonas, su efecto no está tan limitado por el ciclo celular como los modelos de silenciamiento (las histonas canónicas se expresan durante la replicación y el exceso de histonas es controlado para evitar el efecto tóxico, otras variantes se expresan en diferentes momentos) Además, mientras que TSA se usa como fármaco de laboratorio debido a su alta toxicidad in vivo, SAHA ya está aprobado para uso clínico. Utilizamos tres inhibidores de deacetilasas de histonas: TSA, SAHA y VPA. El tratamiento de las células A549 con TSA y SAHA provoca un aumento en la unión de CDDP y DOX. VPA no tiene efecto. Estos resultados concuerdan con el efecto de estos inhibidores sobre la acción de CDDP reduciendo la viabilidad de células A549: TSA y SAHA (en dosis subletales) potencian la eficacia del CDDP, mientras que VPA no tiene efecto. Esto tiene sentido ya que TSA y SAHA son de la misma familia, y ambas actúan sobre un rango de deacetilasas de histonas muchísimo mayor que VPA. Estos resultados confirman los obtenidos anteriormente, que la reducción en la compactación de la cromatina afecta a la unión de fármacos al ADN y además se puede relacionar con el aumento de eficacia de estos fármacos. Este tipo de combinación (de iHDAC y CDDP o DOX) ha demostrado ser útil en el tratamiento del cáncer en muchas ocasiones. Sin embargo, dado que estos medicamentos tienen muchos efectos en las células, la potenciación se ha explicado por otros mecanismos. Por ejemplo por cambios en los patrones de expresión, en la vía de apoptosis, en estado redox, etc. Nuestros resultados indican que la potenciación de la eficacia de fármacos que se unen al ADN como resultado de su combinación con iHDAC podría explicarse al menos parcialmente por la capacidad del iHDAC para reducir la compactación de la cromatina y, posteriormente, aumentar la accesibilidad al ADN para la unión de fármacos.

CONCLUSIONES

I. La reducción de la compactación de la cromatina por manipulación genética de los niveles de histona 4 en un modelo de levadura mejora la unión de fármacos al ADN.

II. La reducción de la compactación de la cromatina, producida por la mutación en el gen zmym2, está relacionado con un aumento de los fármacos anticancerígenos que se unen al ADN en un modelo de células madre embrionarias (ESC) de ratón de línea parental CJ7.

III. En la línea celular de adenocarcinoma de pulmón humano A549, el silenciamiento de CAF-1 y CASP8AP2, que reduce la formación de nucleosomas, aumenta la unión de DOX al ADN.

IV. En la línea celular de adenocarcinoma de pulmón humano A549, el uso de iHDAC TSA y SAHA, que disminuyen la compactación de la cromatina, aumentan la unión de los medicamentos contra el cáncer al ADN y, por tanto, aumenta su eficacia terapéutica.

Índice

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1. ABSTRACT

DNA binding drugs play a major role in cancer therapy. However, their success rate is far from optimal, therefore it is essential to develop new strategies and approaches to enhance patient survival. Drugs that act by binding to DNA face difficulties to bind to DNA once inside the cell. For example, only 1% of CDDP (cisplatin) binds to nuclear DNA. Genomic DNA of eukaryotic cells is packaged in a nuclear complex associated with proteins and RNA called chromatin. Processes like transcription or replication require access to genomic DNA, in which chromatin serves as a regulatory platform by limiting access to DNA. My goal is to explore whether chromatin has a similar effect on drug-DNA binding. Previous results obtained in our group show that Ag_3-AQCs (Atomic Quantum Clusters of 3 atoms of silver) reduce chromatin compaction and improve the efficacy of drugs like oxaliplatin, carboplatin, gemcitabine, CDDP and DOX (doxorubicin). We explored whether this mechanism is specific or if a reduction in chromatin compaction can improve DNA accessibility to drugs and, therefore, chemotherapeutic medicaments efficacy. To test this hypothesis, we quantified CDDP by ICP-MS (induced coupled plasma mass spectrometry) and DOX (by flow cytometry and confocal microscopy) binding to DNA in various models of cells with reduced chromatin compaction. First, we compared two murine ESCs (embryonic stem cell) from CJ7 parental line. These two lines only differentiate in an insertion in zmym2 gene, resulting in reduced chromatin compaction. I also analyzed the effect of a partial depletion of histone H4 in a yeast model. Then, I wanted to test a model with potential clinical application. CASP8AP2 (Caspase 8 associated protein 2), a transcriptional regulator of histone genes and CAF-1 (Chromatin-Assembly Factor 1), a histone chaperone that specifically deposits newly synthesized H3-H4 histones onto replicating DNA,were silenced in A549 lung adenocarcinoma cell line using

siRNAs. Lastly, I studied the effect of iHDACs (histone deacetylase inhibitors), extensively studied and in clinical use in cancer treatment. I selected three iHDACs: trichostatin A (TSA), suberanilohydroxamic (SAHA) and valproate (VPA) with different HDACs targets. Both ESC and yeast models, confirm the increased levels of CDDP and DOX binding to DNA in cells with reduced chromatin compaction compared with wild types with rise of almost 400 % and 300% of CDDP and DOX, respectively, binding to DNA in yeast model. CAF-1 silencing results in an increase of 50 % of DOX binding, while CASP8AP2 silencing only modifies DOX binding by 10 %.iHDACs TSA and SAHA increase CDDP and DOX binding to DNA, while VPA does not. Increasing of the binding of the drugs also increase their effect on cell viability. Thus, potentiation of the efficacy of anticancer medicaments, suchs as CDDP and DOX, by iHDACs TSA and SAHA could be at least partially explained by the reduction in chromatin compaction, that results in increased binding of anticancer drugs to DNA.

2. INTRODUCTION

2.1 CANCER AND ITS TREATMENT

Cancer is the second leading cause of death worldwide. It consists of a group of diseases that are characterized by uncontrolled proliferation of cells and the ability of said cells to invade other parts of the body. The transformation of a normal cell into a cancer cell is the result of multiple genetic mutations and epigenetic modifications that lead to mayor changes in cell biology. [1] Hanahan and Weinberg describe 6 changes in cell biology as hallmarks of tumor development: ability to sustain chronic proliferation, evasion of growth suppressors, resistance to cell death, maintained replicative immortality, induction of angiogenesis, and activation of invasion and metastasis.[2]

Cancer treatments primarily comprise surgery, cytotoxic chemotherapy, targeted therapy, radiation therapy, endocrine therapy and immunotherapy. Chemotherapy is one of the prevalent methods used to treat malignant tumors and includes more than 100 types of agents whose mechanism usually alteration of the cell cycle and produce kjcell death. Conventional chemotherapy includes antimetabolites, alkylating agents and platinum derivates, topoisomerase inhibitors and poisons, antitumor antibiotics and antimicrotubular agents.

Antimetabolites (pyrimidine antimetabolites, like 5-fluorouracil (5-FU) cytosine arabinoside (ara-C), capecitabine and gemcitabine; antifolates such as methotrexate (MTX); and purine nucleoside analogues, fludarabine and cladribine). Their main mechanism of action involves the inhibition of enzymes required during DNA or RNA synthesis; hence they act during the S-phase.

Alkylating agents (like cyclophosphamide, ifosfamide, temozolomide dacarbazine, chlorambucil, carmustine) and platinum derivatives (cisplatin, carboplatin, oxaliplatin). Their common mechanism of action is the alkylation and subsequent formation of covalent adducts with DNA. This leads to a malfunction of DNA synthesis machinery and to apoptosis.

Topoisomerase I inhibitors (camptothecin, irinotecan and topotecan) and Topoisomerase II trap the Top -DNA covalent complex and convert the enzyme into a cytotoxic covalently-linked protein adduct on DNA preventing DNA replication and generating double strand breaks during G2-phase. Anthracyclins (doxorubicin, epirubicin, mitoxantrone) are antitumor antibiotics that inhibit Topoisomerase II action through DNA intercalation. Other examples of antitumor antibiotics are bleomycin and mitomycin C. Bleomycin is a glycoprotein antibiotic that catalyzes single-stranded and double-stranded DNA damage, for which it requires a transition metal, oxygen, and a one-electron reductant). Mitomycin C is a quinone antibiotic that acts as an alkylating agent.

Antimicrotubule agents include the taxanes (paclitaxel and docetaxel) and the vinca alkaloids (vincristine, vinblastine, vinorelbine, and vindesine). Both act by interfering with the balance between microtubules and tubulin and disrupting the assembly of microtubules (M-phase).[3-11]

Chemotherapy faces three major impediments that limit its efficacy: insufficient amount of drug reaching the target, anticancer drug resistance and drug toxicity due to action of anticancer drugs in normal cells.

2.2 CHEMOTHERAPY LIMITATIONS

2.2.1 **Penetration capacity in tumor tissue**

Firstly, a drug that does not reach a tumor will not be effective. Any drug must be available in its place of action in a sufficient concentration. When administered orally, drug should be uptaken into blood, and poor gut uptake would constitute the first limitation.

Secondly, both in oral and intravenous treatments, chemotherapeutic agents may me metabolized or excreted. After reaching the tumor area, drugs must cross the vessel wall and penetrate the interstitium.

An excess of collagen in the extracellular matrix composition of the tumor can block the diffusion of drugs into the tumor. Furthermore, altered tumor vasculature will have mayor impact in drug penetration. Morphology alterations may include dilation, convolution, abnormal branching patterns and width wall abnormalities such as fenestrations, discontinuity or lack of basement membranes, lack of pericytes and lack perivascular muscle. Tumor blood vessels are not organized into arterioles, capillaries, and venules but share features of all of these structures. Sometimes, tumor cells are even integrated in vessel walls. Blood vessels in tumors show functional differences like an increase of permeability and an increase of resistance to blood flow. Consequently, there is a reduction in delivery of nutrients (and drugs) and in the clearance metabolism breakdown products, leading to hypoxic and acidic regions in tumors. Increased permeability together with a lack of functional lymphatic vessels leads to accumulation of fluids in interstitial space and to an increase of interstitial fluid pressure. Augmented interstitial pressure makes it more difficult for drugs to reach the tumor. [12-16]

Hypoxia and lack of nutrients in tumor cells far away from blood vessels maintain these cells in a quiescent state, less responsive to many anticancer drugs that require active metabolic or proliferating cells. Hypoxia forces cells to relay on anaerobic glycolysis to generate energy. This will trigger an acidification of the tumor intersticium. In acidic conditions basic drugs (like doxorubicin) will protonate, which could interfere with their action in cells. [17-19]

Another important challenge to chemotherapy is blood-brain barrier (BBB) which prevents the entry of most conventional anticancer drugs to the brain. [20]

2.2.2 **Drug resistance**

Drug resistance is still a major problem in cancer therapies and responsible for most relapses, one of the major causes of death in cancer patients[21]. Resistance to chemotherapeutics can be divided in two broad categories, intrinsic or acquired, based on the initial response of the tumor to therapy. Acquired resistance, when the tumor is initially reduced in response to treatment but, during the process of treatment, it becomes resistant and starts to grow again; and intrinsic resistance, when the tumor is resistant before being treated. There are some mechanisms an factors that lead to resistance:1) drug altered transport (uptake, export and sequestration), 2) drug metabolization(activation, inactivation), 3) target alteration, 4) DNA damage response, 5) epigenetic changes, 6) cell death inhibition, 7) microenviroment, 8) presence of tumor heterogeneity an CSCs (Cancer stem cells).

Resistance may be limited to the drugs to which patients are being exposed (resistance to a single agent) or be a simultaneous mechanism of absence of response to multiple drugs with different structures and mechanisms of action (resistance to multiple drugs, MDR). [22-24]

2.2.2.1 Drug transport

Drug resistance can be a consequence of drug transport related to three processes: drug uptake, drug efflux and drug sequestration in an inadequate compartment of the cell.

Uptake can be mediated by simple diffusion (doxorubicin and vinblastine), a facilitated diffusion or active transport (CDDP). Drugs can also act without entering the cell by the interaction with a receptor that initiates a signal pathway. [25,26,14]

Uptake can be reduced by mutations that modify activity or reduce the expression of surface receptors and transporters. For example, resistance to CDDP treatment is mediated, among other mechanisms, by a decreased entry of the drug into the cells. Copper transporter 1 (CTR1) plays an important role in the cytotoxicity of

platinum-derived drugs. CTR1 depletion, the main transporter of CDDP into the cell, results in CDDP resistance. [27, 28]

After being absorbed, drugs might be almost immediately expelled from the cell by a transporter. Reduction of drug accumulation inside cells by increased efflux is one of the most studied mechanisms of cancer drug resistance. It could be either acquired or intrinsic. Transmembrane transporters responsible for the drug efflux are primarily from the ATP-binding ABC transporter superfamily.

Members of this transporter family proteins enable efflux and are important, well-studied regulators at the plasma membranes of healthy cells.

The human ABC transporters facilitate unidirectional translocation of chemically diverse substrates, including amino acids, lipids, inorganic ions, saccharides, metals, drugs, and peptides and proteins. We could highlight three transporters: multidrug resistance protein 1 (MDR1/P-glycoprotein/ABCB1), multidrug resistance-associated protein 1 (MRP1/ABCCs), and breast cancer resistance protein (BCRP/ABCG2); that are implicated in tumor chemoresistance. All three have broad, overlapping substrate specificity, including major cancer chemotherapeutics like taxanes, topoisomerase inhibitors and antimetabolites. MDR1 is overexpressed in many tumors and its expression can also be induced by many anticancer drugs (for example, doxorubicin). MDR1 has been associated with chemotherapy failure in many cancers, including kidney, colon, liver, prostate, lung and breast cancers, as well as leukemias and lymphomas. BCRP has been associated with chemoresistance in breast cancer and leukemias. The MDR-associated proteins (MRPs) family is formed by 13 members, including MRP1 (ABCC1). MRP1 is responsible for pumping out a wide variety of anticancer agents, such as vinca alkaloids, anthracyclines, epipodophyllotoxins, camptothecins, and methotrexate. The overexpression of ABCC1 transporters is associated with resistance in many cancer types, including lung, breast and prostate cancers.ABCG2 can transport both positively- or negatively-charged drugs like mitoxantrone, bisantrene, epipodophyllotoxin,

camptothecins, flavopiridol, anthracyclines and imatinib. ABCG2 overexpression was found in many cancer types including breast, lung cancer and leukemia. ABCG2 is considered a marker of CSCs in some cancers. [21, 29, 30, 22, 31]

Lastly, drugs could be sequestrated in cellular organelles or vesicles such as lysosomes, preventing them from reaching their targets. For example, sunitinibbe comes protonated after entering lysosomes, preventing efflux. [14]

2.2.2.2 Drug metabolism

Drug implication in resistance includes inactivation and reduced activation of drugs. Cell mechanisms to protect against toxic agents encompass reactions such as oxidation, reduction and hydrolysis (phase I reactions) and consumption and conversion (phase II reactions). Many antitumor drugs require metabolic activation to exert their effect (pro-drugs). Loss of specific enzymatic activities involved in this process leads to a decrease in the activation of drugs and, consequently, the development of resistance. For example, the conversion of capecitabine to 5-Fluoracil is mediated by thymidine phosphorylase. Methylations in thymidine phosphorylase gene resulting in inactivation, as a result, leads to resistance to capecitabine. [32-34].

Moreover, inactivation of agents that are active can occur. Cytochrome P450 (phase I) is associated with resistance in breast cancer and docetaxel inactivation. Glutathione S-transferase family (GSTs) is involved in detoxification of hydrophobic and electrophilic compounds. High levels of GST in tumor cells will promote the detoxification of drugs, such as alkylating agents and platinum derivatives, resulting in a reduction in the efficacy of these drugs. GST enzymes can increase the drug resistance directly by the detoxification of anticancer drugs or indirectly by the mitogen-activated protein kinase (MAPK) pathway inhibition in the RAS-MAPK path. [25,35].

2.2.2.3 Alterations in drug targets

Drug response can be affected by alteration of its targets, which will have an impact on this union and, consequently, the effectiveness of the treatment. First, overexpression of drug targets is frequently associated with the acquisition of resistance to treatment. For example, androgen receptor (AR) increased expression has been described in 30 % of patients with prostate carcinoma who do not respond to androgen deprivation therapy. Or 5-FU and pemetrexed, thymidylate synthase inhibitors, which post-transcriptionally upregulate thymidylate synthase expression. [34, 36]

Second, mutations, chromosomic reorganizations or other gene alterations of target can lead to resistance to treatment. For example, structural mutations in tubulin**,** or changes in the abundance of β-tubulin isotypes**,** will be involved in the development of resistance to vinca alkaloids and taxanes (for example in ovarian cancer). Likewise imatinib (tyrosine kinase inhibitor) resistance in chronic myelogenous leukemia (CML) can be caused by mutations, amplification or translocation in BCR and ABL genes**.** This alteration prevents the binding of imatinib with BRC-ABL protein, which has been associated with relapses of gastric tumors. [25,37,38]

Resistance to antitumor drugs can also mediate though reactivation of signaling cascades below the therapeutic target or through activation of a parallel signaling pathway. For example, Trastuzumab is a monoclonal antibody that specifically binds to human epidermal growth factor receptor 2 (HER2). Resistance to therapy with this monoclonal antibody is mainly due to alterations in the HER2 receptor that prevent its binding, as well as to modifications in the PI3K / Akt / mTOR signaling path located downstream of the receiver. Alterations in signaling pathway members such as mutations in the catalytic subunit of the phosphatidylinositol-4,5-bisphosphate 3-kinase alpha isoform (PI3KCA) or mutations of phosphatidylinositol-3,4,5-trisphosphate 3 -phosphatase (PTEN) result in a constitutive activation of the pathway and is liked with resistance to treatment with Trastuzumab.[39,40,29**]**

2.2.2.4 DNA damage response

Many chemotherapeutic drugs damage DNA either directly (platinum-derivates) or indirectly (5-FU, topoisomerase inhibitors). Under normal conditions, lesions in the DNA are recognized by DNA damage response (DDR) factors, which activate the cell cycle control points and direct the repair of the damage or, in the event that it cannot be repaired, cell death. Hence, the ability of a tumor cell to repair DNA can determine resistance to drugs that induce DNA damage.[34]

There is more than one signaling pathway involved in the DNA repair process: repair by base cleavage, nucleotide cleavage, homologous and non-homologous recombination and base mismatch repair. Many cancer types have a dysfunction in at least one DNA damage repair pathway, but lack of one of them can be compensated with the other. This compensation contributes to the emergence of resistance against chemotherapeutic agents. [41,42]

Upregulation of genes involved in DNA repair, like FEN1, FANCG, RAD23B, has been reported in 5-FU resistant human colon cancer cell lines. Also, overexpression of the cross-complement group 1 (ERCC1) repair protein is associated to resistance to CDDP in ovarian cancer cell lines. Furthermore, deficiency in the mismatch repair (MMR) system has been related several anticancer drugs resistance. Similarly, hypermethylation of *MLH1* causes resistance to CDDP and carboplatin. [21,34,43-45]

Another example is DNA damage repair protein O6-methylguanyl DNA methyltransferase (MGMT) which is associated with resistance to nitrosoureas, carmustine and temozolomide in central nervous system tumors. [22]

Due to the importance of DNA damage repair systems in cancer cell response to anticancer therapy, they became targets for therapy themselves. However, resistance against drugs that target DNA repair systems also occurs. For example, poly (ADP-ribose) polymerase 1 and 2 (PARP1 and PARP2) inhibitors will be effective against BRCA1 / 2 protein-deficient cells initially, but after treatment,

secondary intragenic mutations will lead to the reactivation of the function and, thus to resistance. [34,36,44]

2.2.2.5 Epigenetic changes

Alterations in epigenetic modifications, like DNA methylation and histone modification, chromatin remodeling, and noncoding RNA related alterations will contribute to tumor development and the emergence of resistance. Epigenetic alteration participates in the development of other mechanisms of resistance (some of them have already been referred to), including increased drug efflux, enhanced DNA repair, and impaired apoptosis. For instance, methylation and silencing of the MLH1 gene that was previously mentioned. Non coding RNA like miRNA (micro RNA, 21-25 nucleotides) and lncRNA(long non coding RNA, more that 200 nucleotides) can interfere in cell response to anticancer drugs by regulating expression of proteins related with resistance. For example, lncRNA urothelial cancer-associated 1 upregulation enhances Wnt signaling and cell survival in CDDP-resistant bladder cancer cells.[21,29,46]

2.2.2.6 Cell death and survival

The objective of anticancer drugs is cell death. However, numerous intrinsic adaptive responses favor cell survival. Constitutive activation of growth signaling pathways and deregulation of cell cycle control points will be associated with tumor development and resistance to chemotherapeutic agents. For example, alterations of Ras / Raf / MEK / ERK or PI3K / Akt / mTOR pathways are related to resistance. [30,47,48]

Cell death can be performed in three ways: necrosis, apoptosis, and autophagy. Necrosis and apoptosis always lead to cell death, while autophagy could contribute to cell survival or cell death. Most anticancer therapies aim to the induction of apoptosis. The evasion of apoptosis is one of the main characteristics of tumor cells which

contribute both to tumor development and progression and to the emergence of drugresistance. An increase in anti-apoptotic proteins and/or a decrease in pro-apoptotic proteins can allow cells to evade apoptosis. For instance, overexpression of antiapoptotic proteins involved in apoptotic signaling pathways, such as Bcl-2 or Bcl-xL, are involved in resistance to imantinib in CML. Another example is the decrease or lack of expression of Apaf-1, proapoptotic factor is related to evasion of apoptosis in melanoma, leukemia, glioblastoma and gastric cancer. Resistance can also be a result of altered expression of IAPs (inhibitors of apoptotic proteins) such as XIAP (X-linked inhibitor of apoptosis protein) which is overexpressed in acute myeloid leukemia and has been related with poor prognosis. [30, 49-51]

Autophagy is a degradation pathway that degrades cellular organelles and proteins in order to maintain cellular biosynthesis and viability during metabolic stresses such as nutrient deprivation. The role of autophagy in cancer is paradoxical as it functions both as a tumor suppressor pathway and as enabler, facilitating cancer cell survival during metabolic stresses caused by anticancer agents. Indeed, inhibition of autophagy has shown potential improving response to alkylating agents in a leukemia mouse model. [30,34]

2.2.2.7 Tumor microenvironment

Cancer cells are not isolated but surrounded by its microenviroment. In the case of solid tumors, the microenviroment consists of the extracellular matrix, stromal cells (fibroblasts, immune and inflammatory cells and blood vessels). In haematological malignancies, the microenvironment is composed of bone marrow stromal cells, bone marrow endothelial cells, osteoclasts, osteoblasts, macrophages, T cells, etc. Tumor microenviroment also includes several soluble factors like cytokines or growth factors. Crosstalk between cancer cells and microenviroment occurs during cancer initiation, progression and metastasis, and is also involved in resistance development. [34,21, 25]

Tumor microenvironment mediated resistance could happen in several types of therapy, including conventional chemotherapeutic agents, targeted therapies, hormonal therapy and radiotherapy.

Integrins are cell surface molecules that mediate adhesion to the ECM. Overexpression of integrins in cancer cells is associated with chemotherapeutic resistance and increased survival. For example, overexpression of integrin β1 correlates with radiation therapy resistance in head and neck cancer, resistance to lapatinib and trastuzumab in breast cancer, and resistance to erlotinib in lung cancer. [34,62]

The response to antitumor treatment could also be affected by the secretion of soluble growth factors or cytokines. These factors participate in autocrine, paracrine and endocrine activation of oncogenic signaling. Sunitinib is a tyrosine kinase inhibitor, which is used as first-line therapy in renal cancer. Elevated levels of interleukin-8 (IL-8) secretion in this type of tumors leads to resistance to sunitinib. [34,53,54].

Crosstalk between cancer and stromal cells also include exosomes containing certain miRNAs. Namely, in CDDP treated neuroblastoma, cancer cells produce exosomic miR-21, which induces in tumor associated macrophages to secrete exosomic miR-155. When these exosomes are internalized by cancer cells, miR-155 silences TERF1 (that codify a telomerase inhibitor) which results in increased telomerase activity and resistance to anticancer drugs.[21]

2.2.2.8 Epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a process in which epithelial cells change their phenotype to a mesenchymal type. This process plays a role in metastasis and anticancer drugs resistance. In EMT the tumor cells lose their epithelial characteristics and acquire mesenchymal properties. It is a sequential process that involves the loss of cell-cell and cell-matrix adhesion, the loss of cellular polarity and the remodeling of the cytoskeleton, thus acquiring greater motility and invasion capacity. Activation of signaling pathways involved in EMT is related with the sensitivity of tumor cells to treatment. For

example, activation of the Hedgehog signaling pathway is associated with resistance to erlotinib in patients with small cell lung carcinoma. Likewise, ERBB2 (HER2) positive breast tumors activating the Wnt / β-catenin signaling pathway correlates with resistance to treatment with trastuzumab antibody. [29, 55-57]

2.2.2.9 Tumor heterogeneity and tumor stem cells

Cancer heterogeneity plays an important role in cancer progression and treatment failure. Most tumors are composed of different types of cells with varied morphological and molecular characteristics. There are two models used to explain tumor heterogeneity: the stochastical model and cancer stem cell (CSC) model.[58, 59]According to the stochastic model, all tumor cells are equipotent and stochastically proliferate or differentiate. In this model, the imperfection of DNA-replication and increased genetic instability of cancer cells due to impaired DNA-repair mechanism, and other factors (like microenvironment) leads to heterogeneity generation. Then, cells with different phenotypes are subjected to evolutionary processes, that end with the selection of best adapted cells.

On the other hand, cancer stem cell model (CSCs) proposes that a cellular subpopulation with the capacity for self-renewal and differentiation would be responsible for tumor initiation and progression. [27,61,62] These CSCs have stem cells characteristics that makes them less sensitive to chemotherapy (senescence, induction of pathways implicated in stem cell maintenance, overexpression of ABC transporter proteins, overexpression of detoxification enzymes, inhibition of apoptotic pathways, enhanced DNA damage repair capacity, etc). Numerous studies have shown that these two models coexist and are complementary rather than mutually exclusive. In both cases therapy acting upon a heterogenous population will select resistant subpopulations.[22, 27, 59-62]

2.3 CHROMATIN AND DNA ACCESSIBILITY

Conventional chemotherapy is still the first line of cancer treatment and DNA targeted drugs play a major role in cancer therapy. Success rate is far from optimal, thus is essential to develop new strategies and approaches to enhance patient survival. [63,64] Drugs that act by binding to DNA face difficulties once inside the cell to bind to DNA. For example, it is known that in CDDP treated cells only 1% of CDDP inside the cell binds to nuclear DNA. [64] We will focus on the study of this problem using two drugs from different families as models: CDDP and DOX (doxorubicin).

CDDP (Cisplatin, cisplatinum, or *cis*-diamminedichloroplatinum (II)) is a widely used treatment in a variety of cancers including bladder, head and neck, lung, breast, ovarian, and testicular carcinomas, germ cell tumors, lymphomas, and sarcomas. CDDP mechanism of action has been associated to its ability to crosslink with DNA purine bases (forming adducts); interfering with DNA repair mechanisms, inhibiting DNA synthesis and mitosis, causing DNA damage, and inducing apoptosis in cancer cells. Other mechanisms of action have been related with CDDP treatment including induction of oxidative stress, induction of p53 signaling and cell cycle arrest, down-regulation of protooncogenes and anti-apoptotic proteins, and activation of both intrinsic and extrinsic pathways of apoptosis. Unfortunately, many tumors develop resistance and to CDDP treatment, related mainly to three mechanisms: altered drug cellular accumulation (decreased uptake and increased efflux), increased drug cytosolic inactivation and increased DNA repair. CDDP can enter cell by diffusion or be actively uptook through the copper transporter CTR1 (which can be degraded and delocalized in response to CDDP in cytoplasm), CTR2 and OCT2. Efflux of CDDP is mediated by ATP7B, ATP7A or MRP2 transporters. After entering the cell, CDDP may be inactivated by glutathione or metallothionein (GSHn or MT), or may bind to its main cellular target, nuclear DNA. The DNA damage can be repaired by ERCC1 and members of the NER pathway. Beside resistance, CDDP toxicity includes severe kidney problems, allergic reactions, decrease

immunity to infections, gastrointestinal disorders, hemorrhage, and hearing loss have been observed. Combination with other drugs is a good way to overcome drug-resistance and reduce toxicity. [65-68] DOX mechanism of action is more unclear. Anthracyclines passively diffuse through plasmatic and nuclear cell membranes. However, cytosolic DOX forms a complex with the proteasome that is translocated from the cytoplasm into the nucleus. DOX has high affinity for DNA, therefore once inside the nucleus, complex with proteasoma dissociates and DOX intercalates with DNA bases to form adducts. This binding, prevents the activity of DNA and RNA polymerases, consequently blocking DNA and RNA synthesis and triggering apoptosis. Mechanism can also be mediated by alterations of the proteasome (reduction of processing and degradation of regulatory proteins that control cell growth, hence initiating apoptosis), inhibition of topoisomerase II (stabilizing DNA breaks originated by Topo II normal function, inhibiting DNA replication and initiating cell death), generation of reactive oxygen species (produced by degradation in cytochrome p450 and leading to lipid peroxidation) and ceramide accumulation in the endoplasmic reticulum (that leads to the translocation of transmembrane protein c-AMP-responsive element binding protein 3-like1 from the ER to the Golgi apparatus, cleaved by site-1 and site-2 proteases, and protein fragment can translocate into nucleus where it acts as a transcription factor that stimulates the transcription of cyclin-dependent kinase inhibitors, which ultimately blocks cell proliferation. [69-72] DOX is used against breast cancers, sarcomas, leukemias, Wilm's tumors, Hodgkin's disease and non-Hodgkin's lymphomas. Main toxic effect of DOX include cardiotoxicity and secondaries leukemias. Resistance involves ABCB1 (MDR1) and ABCC1 (MRP1) and other transporters (ABCC2, ABCC3, ABCG2, and RALBP1); amplification of TOP2A and of ERCC2 (genes related with ezyme repair of DNA damage). [73,74]

Genomic DNA of eukaryotic cells is packaged in a nuclear complex associated with proteins and RNA called chromatin (figure 1). Fundamental unit of chromatin are nucleosomes, in which 147 base pairs of DNA are wrapped around an octamer of four core

histones (H2A, H2B, H3 and H4). Linker DNA between nucleosomes is bonded to histone H1 to compact the chromatin. DNA binds stably with histones by the interaction of negatively charged phosphate with positive charges of histones (basic proteins). Chromatin heterogeneously coils and compact this basic structure further to higher order states. [75-79]. By limiting the DNA accessibility to nuclear machinery involved in transcription, DNA replication, and DNA repair , chromatin regulates processes like gene transcription or DNA repair. [80] Processes like transcription or replication requires access to genomic DNA in which nucleosomes serve as a regulatory platform. Therefore, chromatin must be dynamic allowing local changes in its structure. This dynamic behavior is achieved by the existence of histone variants and post-translational modifications (PTMs). Histone variants shows different levels of affinity to DNA or specific sites for PTMs or for being recognized by chromatin enzymes like histone chaperones, chromatin remodelers, and histone and DNA modifying enzymes. [77]

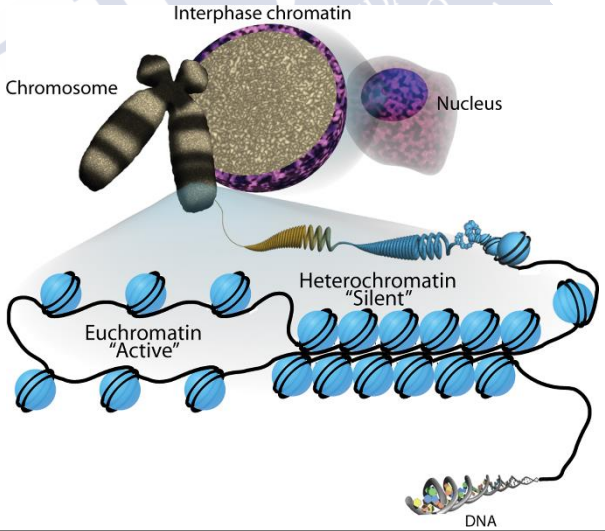

Figure 1. Scheme of chromatin organization showing basic states of compaction. Sha, K. and Boyer, L. A. The chromatin signature of pluripotent cells (May 31, 2009), StemBook, ed. The Stem Cell Research Community, StemBook, doi/10.3824/stembook.1.45.1. http://www.stembook.org/node/585. Creative Commons Attribution License (CC By 3.0)

PTMs like acetylation, methylation, phosphorylation, ubiquitination, modify specific residues of histones as well as other proteins. They can be introduced in free histones or histones within an assembled nucleosome. PTMs can modify histone-histone interactions, DNA-histone interactions and chromatin interaction with other complexes and factors. [81] Of all the known modifications, acetylation has the most potential to unfold chromatin since it neutralizes the basic charge of the lysine. [75]

The assembly, disassembly, and distribution of histones and nucleosomes are controlled by two large families of proteins: histone chaperones and nucleosome remodeling complexes. Histones are synthesized in the cytoplasm and immediately bound by dedicated histone chaperones (necessary for transport, appropriate and specific nucleosome formation, transfer and control of histone degradation). After nucleosomes are formed, chromatin remodelers can change their variant histone composition, compaction level, and position relating to sequence motifs. [78,82]

In the same way chromatin acts as a natural barrier in physiologic conditions for nuclear machinery, we want to explore if it has a similar effect on drug-DNA binding. Previous results obtained in our group show that Ag_3-AQCs (Atomic quantum clusters of 3 atoms of silver) reduce chromatin compaction and consequently improve efficacy of drugs like oxaliplatin, carboplatin, gemcitabine, CDDP and DOX in tumor cells in vitro and of CDDP in mouse models (Figure 2). [83] Since chromatin could act as a barrier to DNA binding drugs, we explored whether mechanism found in AQCs is specific or if a reduction in chromatin compaction can improve DNA accessibility to drugs and therefore chemotherapeutic agents' efficacy. To test this hypothesis, we quantified CDDP and DOX binding to DNA in various models of reduced chromatin compaction.

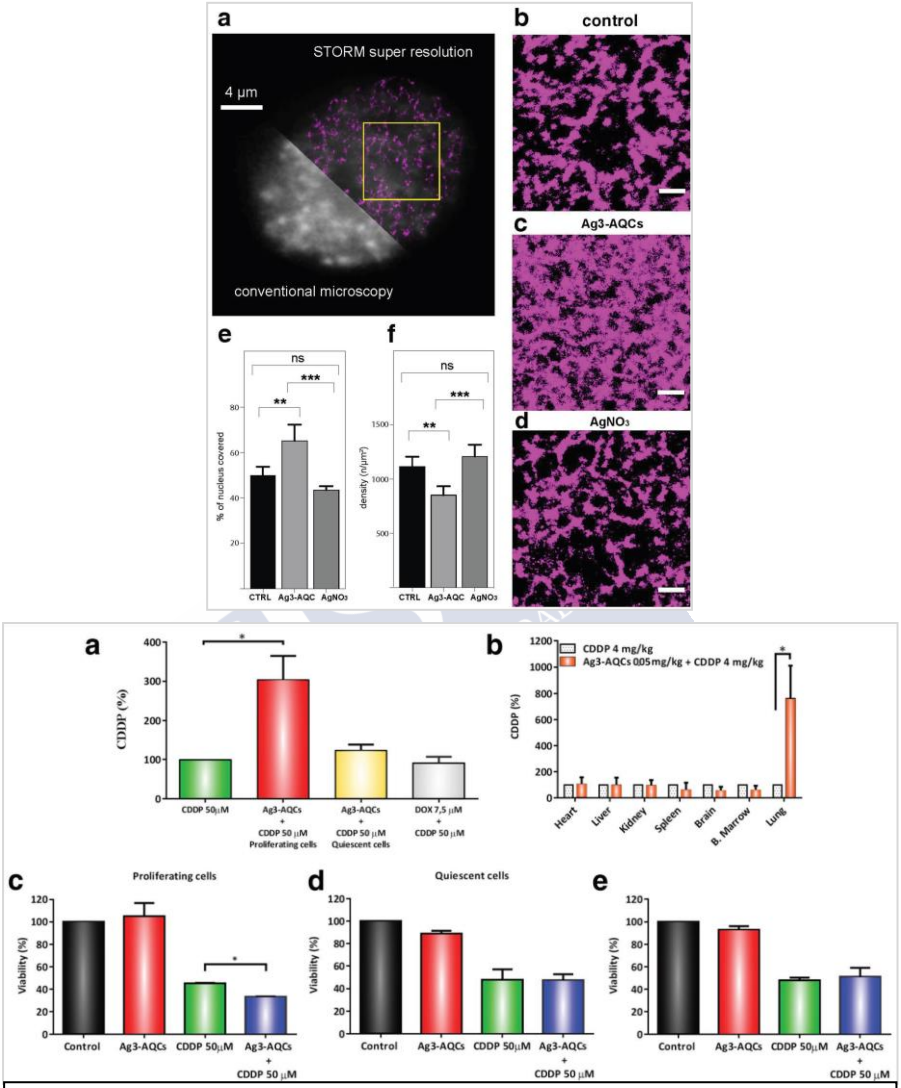

Figure 2. Ag3-AQC effect on chromatin compaction (up) and drug binding (down a) and effect on drug efficacy in vivo and in vitro(down b-e). Figures 2 and 4. Porto, V., Borrajo, E., et al., Adv. Mater. 2018, 30, 1801317. https://doi.org/10.1002/adma.201801317. Figures used under license agreement with John Wiley and Sons and Copyright Clearance Center. License Number 4684091478282.

First, in a collaboration with *Stem Cells & Human Diseases Lab*, we compared CDDP and DOX binding to DNA in two murine ESCs (embryonic stem cell) from cj7 parental line. These two lines only differentiate in the insertion by GeneTrap of a gene that confers resistance to antibiotic g418 in zmym2 gene, resulting in prevention of complete protein translation (Jianlong Wang Lab, Icahn School of Medicine at Mount Sinai Hospital, New York). As a result of this modification, cells exhibit lower levels of chromatin compaction compared with wild type cells that even affect nuclei size. (Figure 3)

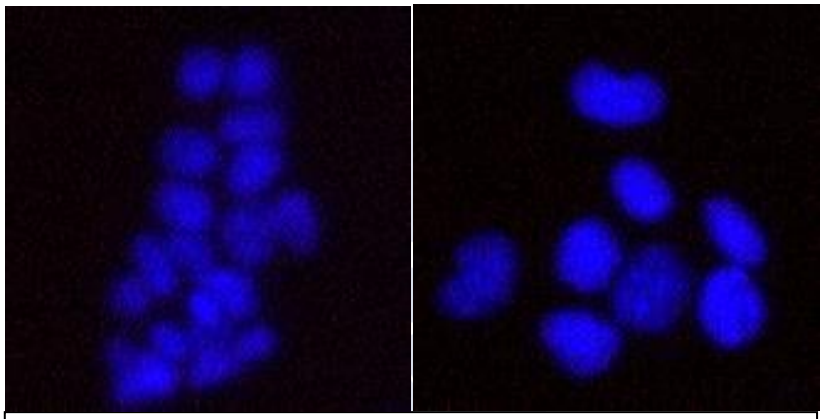

Figure 3. Hoechst dye of left, wild type; and right, KO cells (resistant to g418). Mutant cells shows less compacted crhomatin, and larger nuclear size.

Afterwards, to study the effect in a more controlled model, we analyzed the effect of a partial depletion of histone H4 in a yeast model in collaboration with *DNA Repair and Genome Integrity Group*. As histone availability has shown to affect chromatin compaction both in yeast and humans. We compared two strains of *Saccharomyces cerevisiae* one with normal H4 expression and the other in which the expression of histone H4 was driven by a regulated *tet* promoter. In this strain, H4 expression is induced by treatment with doxycycline, never reaching normal expression levels. This partial depletion leads to defective nucleosome assembly. Since

mutant strain shows altered cell cycle due to difficulties cells found in H4 depletion, nocodazole (that disrupts microtubule assembly/dissasemby dynamics and prevents mitosis) was used in attempt to synchronized cells prior to CDDP or DOX treatment. [84,85]

There are however many differences in yeast response to nucleosome assembly modification compared with humans; [111-113,122] and we wanted to test a model with potential clinical application. Two biological targets related with chromatin formation and maintenance were selected: CASP8AP2 and CAF-1. CASP8AP2 is a transcriptional regulator of histone genes and Chromatin-Assembly Factor 1(CAF-1) is a three-subunit histone chaperone (with subunits p150, p60 and p48) that preferentially deposit newly synthesized H3-H4 histones onto replicating DNA during S phase. In order to reduce its chromatin compaction, adenocarcinoma human cell line A549 was transfected with siRNA that target CASP8AP2 mRNA, chaf1b mRNA (that codifies p60 subunit of CAF-1) or and siRNA without a target in cells. siRNAs are double strand RNAs of small size (in this case 19 base pairs) that interfere with expression by binding and posterior excising target mRNA. [86-89]

Lastly, we studied the effect of some drugs extensively studied and in clinical use in cancer treatment: histone deacetylase inhibitors (iHDACs). iHDACs are inhibitors of histone deacetylases (HDACs), one of main classes of PTM enzymes. Histone deacetylases catalyze the hydrolysis of N-acetyl lysine residues in histones and act in opposition to the action of histone acetyl transferases. Hyperacetylation results in more relaxed chromatin. HDACs are divided into four main classes of zinc dependent enzymes Class I (HDAC 1, 2, 3, 8), Class IIa (HDAC 4, 5, 7, 9), Class IIb(HDAC 6, 10) and Class IV (HDAC 11) and the non-zinc dependent Class III enzymes (NAD+-dependent sirtuins). We selected three iHDACs: trichostatin A (TSA), suberanilohydroxamic (SAHA; vorinostat; Zolinza®) and valproate (VPA). TSA and SAHA are hydroxymates that inhibit all HDACs. VPA is a short-chain fatty acid that only inhibits Class I and IIa HDACs. As in previous models we studied CDDP and DOX binding to DNA in A549 cell line. [90-94]

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3. OBJECTIVE

To study the effect of chromatin compaction and its potential pharmacological modifications in order to increase the DNA binding medicaments therapeutic activity.

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4. MATERIALS AND METHODS

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich.

Three different cell types were used: a yeast model, a mouse ESC model, and A549 human lung adenocarcinoma (for silencing models and IHDAC treatment model).

CDDP was prepared in PBS at stock 1mg/mL and DOX was resuspended in DMSO (Dimethyl sulfoxide) at 1mg/mL too.

4.1 ESC MODEL

4.1.1 ESC model cell lines and culture

All experiments with ESC model were performed in collaboration with Yara Souto Becerra from Stem Cells and Human Diseases group (CiMUS, Santiago de Compostela) under the supervision of Miguel Fidalgo Pérez. ESC model consists of two mouse embryonic stem cell lines from CJ7 parental line. One is wild type (WT) and the other has knockout of Zmym2 gene (KO) obtained after insertion by GeneTrap of a gen for G418 antibiotic resistance in Zmym2 gene. KO line was created by Dr. Jianlong Wang Lab (Icahn School of Medicine, Mount Sinai Hospital, NY). Cells are maintained in Dulbecco's Modified Eagle Medium (10-103-CVR, Corning) supplemented with 10 % FBS south American (Fisher Scientific), L-glutamine, penicilin/streptomycin, uridine, adenosine, cytidine, thymidine, guanosine, MEM non-essential aminoacid solution (Inqualab), 2-mercaptoethanol, and LIF (leukemia inhibitor factor). Before culture, cell dishes were incubated with jelly 0.1 % for 30 minutes at 37 ºC. Cells were incubated in a humidified incubator at 37 °C with 5 % CO2 and grown in 6-well culture plates with daily medium replacement. For

subculturing, the medium was removed, and cells were washed with phosphate buffered saline (PBS); trypsin/EDTA (Gibco) was added and incubated for 5 min at 37 ºC to induce cell detachment. Finally, the cells were suspended in a culture medium and transferred into fresh dishes at a ratio of 1:3 - 1:6. All procedures using ECs were performed under sterile conditions in a laminar air flow hood.

4.1.2 **DNA-drug binding in ESC model**

4.1.2.1 CDDP binding in ESC model

CDDP binding to DNA was direct quantified as ratio of Pt content/ phosphorus content in DNA samples of cells treated with CDDP. $6X10^5$ ESCs were seeded in 60 mm diameter plates. After 12-16 hours cells were treated with 50 μM for 6 h in ESC described complete medium. After the treatment, the cells were washed with PBS, trypsinized and centrifuged. The supernatants were removed, and the cell pellets were stored at -20°C overnight. From this point, DNA was extracted using Nucleospin Tissue kit (Macherey-Nagel) with an extra addition of RNAse digestion suggested as optional in kit protocol.

DNA was resuspended in kit elution buffer and its concentration was determined using a NanoDrop 2000 spectrophotometer (Thermofisher). Then, elution buffer was removed using the speed-vac (H_2O conditions, 37 ºC), and after DNA was resuspended in 0.2 mL of 65 % HNO_3. Finally, the amount of platinum and phosphorus was determined by mass spectrometry using an ICP-MS BRUCKER 820-MS with a low-flow glass Micromist nebulizer and a double-pass spray chamber with a Peltier cooling (3 °C) and quartz torch (Bruker Corp.). ICP-MS was performed by Verónica Piñeiro of Unidade de Análise Elemental (CACTUS, Lugo).

4.1.2.2 DOX binding in ESC model

DOX binding was measured using its own fluorescence in two ways: quantifying DOX fluorescence in cells by flow cytometry and quantifying nuclear DOX fluorescence by confocal microscopy.

For flow cytometry assay, $500x10^3$ cells were seeded in 6-well plates. 12-16 hours later, cells were treated with 7.5 μM DOX for 4 h. Next, cells were collected, washed twice with PBS suspended in 200 μl of cold PBS and analyzed on the Guava EasyCyte flow cytometer using the InCyte program.

For microscopy approach 100×10^3 were seeded in 25 mm^2 confocal special plates. After 12-16 h, cells were treated with DOX (7.5 μM) for 3 h, then washed with PBS and PBS+10 % FBS added and images were obtained using confocal microscopy Leica TCS SP8 (Images were obtained by Marta Picado Barreiro from Confocal Service of IDIS, Santiago de Compostela). Maximum projections of images were obtained using Leica software LAS X, and then nuclear fluorescence was quantified with ImageJ (RawIndent).

4.2 YEAST MODEL

4.2.1 **Yeast model cell lines and culture**

All yeast model experiments were performed in collaboration with Tomás Lama Díaz from DNA Repair & Genome Integrity Lab (CiMUS, Santiago de Composela), under the supervision of Miguel González Blanco. Two strains were used in yeast models. A partially H4 depleted strain: ΔH4 (ΔH4); and a strain with normal expression of H4, WT (WT). Partially H4 depleted strain, ΔH4, was created by Prado and Aguilera (Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Seville, Spain) [85] and is a null mutant for the two copies of the histone H4 gene (*hhf1*Δ *hhf2*Δ) in BY4741 strain and contains a plasmid (p413TARtetH4) expressing *HHF2* under the control of the

bacterial *tet* promoter. This plasmid harbors copies of the *tetR'-SSN6* and *tetR-VP16* fusions, which positively regulate the *tet* promoter in response to doxycycline in a dose-dependent manner (used in 5 μg/mL or 0.25 μg/mL). This strain can grow in a medium without histidine. The other strain, WT is a strain with the same background (BY4741) as ΔH4, that was transformed with plasmid pRS413 to confer it the ability to grow without histidine supplementation. Synthetic Complete medium minus histidine (SC-his) was used. Unless otherwise specified, the medium was supplemented with 5 μg/mL of doxycycline. Cells from -80 ºC frozen stocks were plated in petri plates containing SC-his agar medium and 5 μg/mL of doxycycline. After 2-3 days, some colonies were selected and transferred to an Erlenmeyer flask containing SC-his liquid medium. Afterwards, samples were collected (for protein extraction and cell cycle analysis) and cells were synchronized with nocodazole (15 μg/mL for 2 hours). The cell number was counted using a Neubauer chamber and cultures were normalized to obtain the same density in both cultures before performing experiments. Sterile conditions were obtained by using a gas burner for yeast manipulation.

4.2.2 **H4 expression in yeast model**

In order to check that our cultures contain strains of interest, H4 levels were studied using two methods: protein extraction and western blot, and immunostaining of H4 in cells and quantification by fluorescence microscopy.

For western blotting, yeast samples were collected before the treatment from the same cultures that were used in the experiment (WT, ΔH4 treated with 0,25 or 5 μg/mL of doxycycline); then, TCA extraction was performed. Briefly, yeast pellets were pre-treated with cold 10 % TCA and 1 vol of acid-washed glass beads was added. After, BeadBeater was used at full speed 2x30 s. Supernatant was then collected and LDS-loading buffer and Tris base, added. Samples were boiled for 5 min and centrifugated 10 min at maximum speed. Supernatant were collected and the total

protein was quantified using colorimetric method. Extracts were normalized and 40 µg of protein/sample were loaded in a 4-20 % precast gel, and electrophoresis was performed. Proteins were transferred to PVDF membrane using semidry transference method. Membrane was blocked with 5 % milk. And antibodies used were: Anti-histone H4 antibody at 1:1000 (ab10158, Abcam) and Pgk1 (abcam) followed by anti-rabbit HRP secondary antibody 1:3000 (abcam).

For H4 inmunostaining, cells grown overnight (WT and ΔH4) with 5 or 0.25 µg/mL of doxycycline were synchronized with alpha factor (for G1 arrest), then cells were pelleted. 0.1 vol. of 37 % formaldehyde (stabilized with about 10 % methanol) was added to the samples and then incubated O/N at 4 ºC. Pellets were washed three times in 0.1 M-KPi buffer pH 6.4 and once in spheroplasting buffer (0.1 M KPi pH 7.4, 1.2 M sorbitol, 0.5 mM MgCl2). Cells were resuspended in 0.2 ml of spheroplasting buffer. 4 µl of β-mercapto-ethanol diluted 1:10 (in water) was added and incubated at 30 ºC for 15 min. 10 µl Zymolyase solution (Zymoliase 100T in spheroplasting buffer) was added to the cells, incubated at 30 ºC until spheroplast formation (under microscope, spheroplasts appear dark whereas non-spheroplasted cells are refractile). Cold spheroplasting buffer is used to stop the reaction and then wash cells. For antibody staining 10 well slides were used. After being coated with 0.1 % polylysine, a drop of cells was added, dried and fixed with MeOH-acetone at -20 ºC. After warming, BSA-PBS was added and incubated at RT for 30 min. Afterwards, anti-histone H4 1:200 (abcam) diluted in BSA-PBS was added. Following ON incubation at 4 ºC wells were washed 4 times with BSA-PBS and antirabbit- Alexa 594 antibody (fluorescent) diluted in BSA-PBS, added. Next, samples were incubated in dark 1 hour at RT and washed four times in BSA-PBS. Then, pd-DAPI mounting medium was used and coverslip sealed with nail polish. Images were obtained using confocal microscopy Leica TCS SP8 (Images were took by Marta Picado Barreiro from Confocal Service of IDIS, Santiago de Compostela). Maximum projections of images were

obtained using Leica software LAS X, and then ratio of histone fluorescence/DNA staining was quantified with ImageJ.

4.2.3 **DNA-drug binding in H4 partially depleted yeast model**

For both CDDP and DOX binding experiments, cells were cultured ON, then synchronized with nocodazole and treated with 0.2 mM of DOX for 2 hours or 0.5 mM of CDDP for 4 hours.

Cells treated with DOX were then washed and resuspended in PBS. Flow cytometry was performed with BD Accuri™ C6 Plus and results were analyzed with BD software.

CDDP treated cells were washed and frozen at -20º C. Then DNA was extracted using Gene Jet genomic DNA Purification kit (Thermo Fisher Scientific) following kit protocol with two modifications. 20 µL of RNAse 20 mg/mL and 5 mg/mL of zymolyase 100T were added in first step. Next, DNA was run in a 0.7 % agarose gel to check RNA presence and, when necessary, second digestion of DNA followed by etanol-acetic precipitation was performed. Afterwards, samples were processed and analyzed as previously described in ESC model).

4.2.4 **Cell cycle of H4 partially depleted yeast model**

Samples from experiments were taken before and after nocodazole treatment, and after DOX or CDDP treatment. Right after cells were put in ice, resuspended in 1 ml 70 % ethanol and stored at 4 °C ON or until required. Then cell pellets were washed with 50 mM Tris pH 8. Next, they were resuspended in 1 ml 50 mM Tris pH 8 with 10 µl of 10 mg/ml RNaseA (DNase-free). Later, cells were incubated 4 h-overnight at 37 °C. Afterwards, pellets were resuspended in 0.5 ml of 5 mg/ml pepsin freshly dissolved in 50 mM HCl and incubated in a 37 °C water bath for 30 min. Finally cells were resuspended in 0.5 ml 50 mM Tris pH 8 and 50 µl of cells were transferred into 0.5 ml 50 mM Tris pH 8 with 1 µM SYTOX Green, mixed and sonicated.

Flow cytometry was performed with BD Accuri™ C6 Plus and results analyzed with BD software.

4.3 HUMAN CELL MODELS

Silencing and iHDAC models were performed with Human lung adenocarcinoma cell line (A549) obtained from DMSZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. They were grown in adherent monolayer and maintained in Dulbecco's Modified Eagle Medium (low-glucose, D6046, Sigma). The medium was supplemented with 10 % fetal calf serum and 1 % (v/v) L-glutamine, penicillin and streptomycin (Gibco, Thermofisher). Cells were incubated in a humidified incubator at 37 °C with 5 % CO2 and grown in 75 mm^2 culture flask to approximately 70-80 % confluence. For subculturing, the medium was removed, and the cells were washed with phosphate buffered saline (PBS); trypsin/EDTA (Gibco) was used to induce cell detachment. Finally, the cells were suspended in culture medium and transferred into fresh flasks at a ratio of 1:4 - 1:10. For the experiments cells were counted using the automated cell counter TC20 from Bio-Rad. Cells were stored frozen with complete growth medium supplemented with 10 % DMSO (Sigma, D2650) at liquid nitrogen vapor temperature. All procedures using human cells were performed under sterile conditions in a laminar air flow hood.

4.3.1 **Silencing models**

Two proteins related to nucleosome formation were assessed: CASP8AP2 and CAF-1 (p60 subunit). Both models were performed in A549 human lung adenocarcinoma cell line using Dharmacon siRNAs and DharmaFECT transfection reagent. During DMEM, the medium previously described was used without antibiotic supplementation.

4.3.1.1 Expression after silencing

A549 cells were plated in 6-well plates. After 12 hours transfection with 100 nMsi RNA and 5 µL/150000 cells DharmaFECT reagent was performed following Dharmacon protocol. 5 different conditions were prepared: untreated cells, non-targeting (NT) siRNA transfected cells, GAPDH siRNA transfected cells, CHAF1B siRNA transfected cells and CASP8AP2 transfected cells. Since CAF-1 and CASP8AP2 have an effect on cell proliferation, cell number and transfection was adapted (1.5x105 cells in untreated, NT and GAPDH; and 3x105 in CAF-1 and CASP8AP2), and after 6 hours the transfection medium was replaced by a fresh medium. 48 hours later, transfection cells were collected and placed on ice. Samples were washed twice with cold PBS and frozen at -80 ºC. RNA was isolated using the kit NucleoSpin ARN (Macherey- Nagel, Düren, Germany) following the manufacturer instructions. The RNA concentration was quantified using a spectrophotometer (Nanodrop 2000) and then RT-PCR (Reverse Transcription- Polymerase Chain Reaction) was performed as described below. 500 ng of RNA samples were prepared in 12.5 µL of H2Omq and mixed with 1.5 µL dNTPs (Invitrogen) 50 nM and 5 µL of random primers 50 nM (Thermo Fisher). They are heated to 65 ºC for 5 min. Then 6 µL of 5XBuffer (Invitrogen), 3 µL of 0.1 M DTT (Invitrogen) and 1 µL of RNAse OUT™ Recombinant (Thermo Fisher) are added and mixed. Samples are incubated another 2 min at 37 ºC and 1 µL M-MLV reverse transcriptase enzyme (Thermo Fisher Scientific) is added (30 µL final volume). Then samples were incubated at 25 ºC for 10 min, then at 37 ºC for 50 min and at 70 ºC for 15 min. cDNA obtained is then used or stored at -20ºC.

cDNA relative quantification was then meassuered using RTqPCR (Real Time PCR). TaqMan Realplex System was used. In this system a labeled probe is specific for every sequence we want to measure. For each reaction we mixed 2 µL of cDNA, 10 µL of Taqman® gene expression master mix (Thermo Fisher) and 1 µL

of one of the probes (MGB Family, Applied Biosystems, Thermo Fisher) with 7 µL of sterile H2Omq. Five houskeeping genes were tested: GUSB (Hs00939627_m1), TFRC (Hs00951083_m1), TMED5 (Hs00211349_m1) and GCLM (REF) . Probes for silencing genes were GAPDH (Hs02758991_g1), CAF-1 (Hs01123302_m1) and CASP8AP2 (Hs01594281_ms). Reaction was performed at 50 ºC for 2 min, then 95 º for 10 min; next 40 cycles of 95 ºC for 15 s, 60º C for 15 s and 72 ºC for 1 min. Real time PCR was performed in AB StepOnePlus™ Real-Time PCR System (Thermo Fisher) and data was analyzed using its software. Relative gene expression was determined using $2^{-\Delta\Delta Ct}$method using GUSB as endogenous control for calculations.

4.3.1.2 DNA-drug binding in silencing models

Only DOX treatment was studied in these models, and DOX-DNA interaction was measured as DOX content in cells by flow cytometry. 5x103 a549 cells were cultured in 96-well plates. CAF-1 transfection was performed after 12 h with 100 nM siRNA of chaf1b or NT and 0.5 µL/well of DharmaFECT. Untreated cells (not transfected) were used to check viability effect of transfection. Medium was changed after 6 h. 48 h after transfection 7.5 µL of DOX was added and cells were incubated for 4 h. In CASP8AP2 silencing model 3x103 cells/ well were cultured and after 12 h, transfection was done with 100 nM siRNA and 0.3 µL/well of DharmaFECT reagent. Medium was replaced after 6 h and DOX treatment was added 72 h after transfection for 4 h (7.5 µM) or 24 h (0.5 µM). After DOX treatment, cells were collected, washed twice with PBS resuspended in 200 µl of cold PBS and analyzed on the Guava EasyCyte flow cytometer using the InCyte program.

4.3.2 **IHDACs**

Three iHDACs, TSA, SAHA and VPA, and one DNA metiltransferase inhibitor, procaine hydrochloride, were tested. These drugs were bought form Sigma Aldrich in powder form and

prepared as follows. TSA (Trichostatin A, Sigma aldrich) was suspended in DMSO at 50 μM, SAHA (suberanilohydroxamic acid) was suspended in DMSO at 9.4 mM and VPA (vaproic acid) and Procaine hydrochloride were prepared in sterile H2Omq at 20 M and 10 M, respectively.

4.3.2.1 Viability effect of iHDACs

CDDP and DOX cytotoxicity measurement: first, we tested the effect on cell viability of CDDP and DOX alone. 4x103 A549 cells were cultured in 96-well plates. 24 h later the medium replaced with complete medium containing different concentrations of CDDP (7.5-100 μM) or DOX (0.125-50 μM) for 24. Then, 10 μl of MTT solution (5 mg/ml) were added to each well and incubated at 37 °C protected from light. 4 h later, 100 μl of solubilization solution (SDS/0.1NHCl) were added and samples were incubated for 18 h at 37 °C. Absorbance was measured at 570 nm using a CLARIOstar® microplate reader.

Cytotoxicity of combination of CDDP and iHDACs was tested in two modes: short pretreatment for 4 h with iHDAC followed by treatment with CDDP for 24 h or cotreatment of iHDAC+CDDP for 24 h.

In the first setting, cell viability was quantitated by flow cytometry using the Guava ViaCount Reagent (Millipore). 6x104 A549 cells were cultured in 12-well plates. Then, cells were treated with TSA 33 nM, SAHA 1.25 μM, VPA 200 μM and procaine hydrochloride 50 μM, in complete medium or nothing (just medium replaced) for 4 hours. After that, medium was replaced with 50 μM CDDP in complete medium or complete medium alone. This way, conditions tested were: iHDACs (or procaine hydrochloride) alone, CDDP alone or combination od iHDACs and CDDP. After 24 h, cells were collected, washed with PBS and suspended in 500 μl of PBS. To prepare stained samples, the cell suspension was mixed with the Guava ViaCount Reagent (Millipore) following the manufacturer's instructions. Stained cells

were analyzed on the Guava EasyCyte flow cytometer (Millipore) using the Guava ViaCount software.

For cotreatment experiments, 4x103 A549 cells were seeded in 96-well plates. 24 h later they were treated with TSA 50 nM, SAHA 1.25 μM, VPA 200 μM and procaine hydrochloride 50 μM, CDDP 50 μM or combinations of CDDP with the other. After 24 h MTT assay was performed as previously described. A dose-response combination experiment was also performed in the same conditions as the above cells were treated with: TSA (25 nM, 50 nM or 75 nM), SAHA (0.75 μM, 1.25 μM or 2.5 μM), VPA (100 μM, 200 μM, 400 μM or 600 μM), procaine hydrochloride (25 μM, 50 μM, 100 μM) or CDDP (50 μM) alone or the combination of iHDACs in all this concentrations with CDDP and MTT assay was performed.

DOX combination was tested with TSA. In cotreatment conditions, cells were treated with TSA 50 nM, 100 nM or 300 nM and DOX 1-30 μM.

4.3.2.2 DNA-drug binding in iHDAC treated cells

4.3.2.2.1 CDDP binding in iHDAC treated cells

CDDP binding to DNA was measured by ICP-MS. 5x105 A549 cells were cultured in 60 mm dishes and 24 h later treatments were applied: untreated, treated with iHDACs (TSA 300 nM, SAHA 2 μM and 4 μM and VPA 200 mM), CDDP (50 μM) or combination of iHDACs and CDDP. After the treatment, cells were collected and processed as described for CDDP quantification ESC model.

4.3.2.2.2 DOX binding in iHDAC treated cells

DOX binding was measured using its own fluorescence by flow cytometry and and confocal microscopy.

For flow cytometry assay, $30x10^3$ cells were seeded in 24-well plates. 24 hours later, cells were treated with TSA (50, 100,

300 nM,), SAHA (2, 5 and 10 μM), VPA (200, 500 and 600 μM) for 24 hours and then treated with 7.5 μM DOX for 4 h. Next, cells were collected, washed twice with PBS suspended in 200 μl of cold PBS and analyzed on the Guava EasyCyte flow cytometer using the InCyte program.

For microscopy approach $30x10^3$were seeded in 25 mm^2 confocal special plates. After 24 h, cells were treated with TSA 0, 50 or 300 nM for 24 h. Then cells were treated with DOX (7.5 μM) for 4 h, then washed with PBS and PBS+10 % FBS added and images were obtained and processed as in ESC model.

4.3.2.3 Cell cycle

Additionally, DNA content of cells treated with TSA was quantified to know the effect that treatment has in cell cycle. 30x103 A549 cells cultured in 24-well plates were treated with 0, 50 or 100 nM of TSA for 24 h. After the treatment, cells were collected, washed twice with PBS and fixed in 70 % ethanol overnight. Next, cells were washed twice with PBS, and incubated for 30 min in the dark in 0.5 ml propidium iodide (0.1 mg/ml). The DNA-stained cells were analyzed using a Guava EasyCyte flow cytometer using the InCyte program (Millipore).

4.4 STATISTICAL ANALYSIS

All statistical analyses were performed with GraphPad Prism Version 13 software (GraphPad Software, Inc., La Jolla, USA). The differences were considered significant for $p < 0.05$. Results are expressed as mean ± standard deviation. Means were compared using one coiled t of Student analysis in two samples comparison when samples present gaussian distribution and both populations have same variance. When more than one group mean is compared with gaussian distribution and homocedastic populations, one way-ANOVA followed by Dunnet multiple comparison analysis was performed. Two normality test were used to test gaussian distribution D'Agostino-Pearson (when samples were big) and

Shapiro-wilk (for small samples) and the Browne-Forsythe test was used to test equally of variances. When normality was rejected, Mann-Whitney assay was performed. When populations were heterocedastic, Welch correction of t-Student and ANOVA were used. In samples with $N > 50$, parametric analysis were performed even when normality was rejected based on central limit theorem.

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5. RESULTS

Chromatin could act as a barrier to DNA binding drugs. To study if a reduction in chromatin compaction can improve DNA accessibility to drugs and therefore chemotherapeutic agents' efficacy, we quantified CDDP and DOX binding to DNA in various models of reduced chromatin compaction. Our first approach was using an embryonic stem cell (ESC) model, in which a mutation was introduced to provide resistance to an antibiotic results in reduced compaction of chromatin. After finding a significant increase in CDDP binding that supports the hypothesis, we wanted to test a more defined model. For this, we used a genetically modified yeast strain to reduce histone H4 expression. Results obtained in yeast confirmed those obtained with the ESC model, indicating that reducing chromatin compaction can increase CDDP and DOX binding. Our aim is to study if these results could be applied in cancer treatment. Therefore, we tried to develop a pharmacalogical approach to reduce chromatin compaction. Based on previous data , we focused on the assembly of new nucleosomes during DNA replication. Nucleosome assembly is a complex mechanism that requires, among others, CAF-1. To see the effect of silencing these proteins, we knockdown proteins related with nucleosome assembly. This method is difficult to translate to the clinic so a new approach using iHDACs, drugs that can reduce chromatin and already in use in clinic, was choosed.

5.1 ESC MODEL

First we used a embryonic stem cell from mice. ESC from cj7 parental line was selected as a model since a mutant obtained by the insertion of the gene for resistance to an antibiotic in Zmym2 that disrupts expression of the protein originates a change in chromatin compaction.

5.1.1 **DNA-drug binding in ESC model**

5.1.1.1 CDDP binding in ESC model

CDDP binding to DNA was measured by ICP-MS quantification of elements Pt and P in DNA samples of mutant and wild type ESC cells. Mutant cells, with reduced chromatin compaction, have 10 % more CDDP binded into DNA (Pt/P) than WT. (Figure 4)

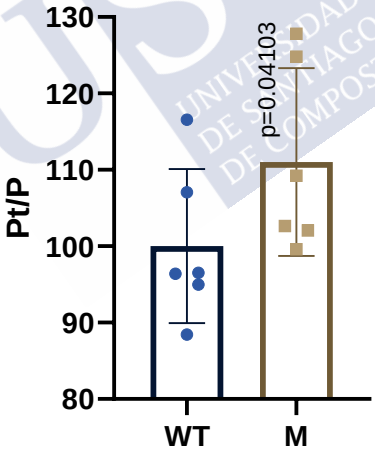


Figure 4. Pt/P content in DNA samples of WT and M (mutant) ESCs treated with CDDP. The data is standardized by calculation of percentage compared to WTcells incubated with CDDP 50 μM(100 %) and shows mean±SD as well as individual data(N=6). Mutant cells show increased Pt/P ratio. p-value of one coiled t-Student analysis.

5.1.1.2 Doxorubicin binding in ESC model

DOX binding to DNA was measured by flow cytometry and confocal microscopy. Mutant cells, with reduced chromatin compaction, show higher DOX content than WT cells in flow cytometry and also higher content of DOX in nucleus in confocal microscopy. A significant increase of DOX content in nuclei of cells with reduced chromatin compaction was observed in this model too. (Fig 5)

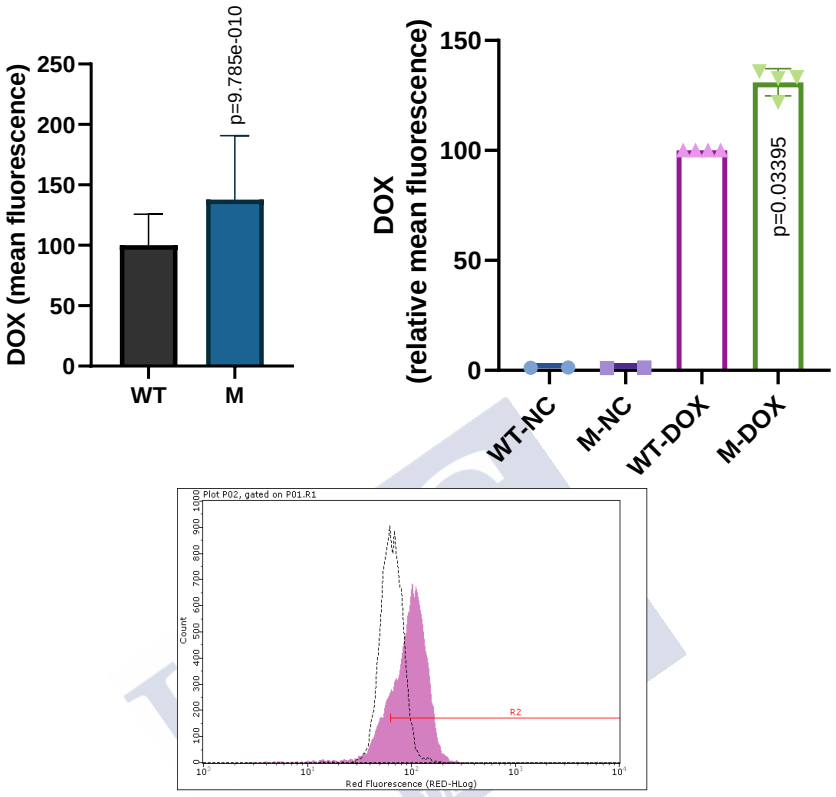


Figure 5. Up left: DOX content in nucleus WT and M cells. Graph shows integrated density of fluorescence intensity of nuclei of cells treated with DOX that show a statistical significant ($p<0,05$, Welch corrected t-student test) increase in doxorubicin binding in mutant cells (with lower chromatin compaction). Data shown as mean±SD of $N>90$. Up right:intracellular DOX content in ESC model measured by flow citometry. Mean fluorescence of cells treated with DOX 7.5 μM. The data is standardized by calculation of percentage compared to WTcells treated with DOX (100 %) and shows mean±SD. Mutant cells show increased DOX content compared with WT. Down: flow cytometry profiles of DOX treated cells WT (transparent) vs. M (pink).

5.2 H4 PARTIALLY DEPLETED YEAST MODEL

In our yeast model chromatin compaction alteration is a direct result of H4 reduced levels. In this model we compared CDDP and

DOX binding in two strains of *Saccharomyces cerevisiae.* Yeast strain with partially depleted H4, t::HHF2, *(*ΔH4*)* is a null mutant for the two copies of H4 gene and contains a plasmid (p413TARtetH4) that express H4 under the control of bacterial *tet* promoter. This means that H4 expression is regulated in this strain by the promoter in a dose manner response to tetracycline doxycycline (however, levels of H4 expression in mutants do not reach wild type levels even with a high dose). The other strain, with normal h4 expression (WT), is a strain with same background BY4741 transformed with plasmid pRS413. These strains will be referred as ΔH4and WT from now on.

Since doxycycline regulates promoter in a dose dependent manner, two different concentrations of doxycycline were used in ΔH4 5 or 0.25 μg of doxycycline/mL and 5 μg/mL in WT. The mutant strain shows an altered cell cycle due to difficulties cells found with H4 depletion, then, nocodazole (microtubule desestabilizing and subsequently antimitotic agent) was used to synchronized cells prior to CDDP or DOX treatments.

5.2.1 **H4 expression in H4 partially depleted yeast model**

First, to check that the selected colonies were trully the ones that contain both depletion and plasmid with inducible gene, protein expression was assessed by western blot and immunofluorescence analysis. H4 was reduced in mutant (ΔH4) versus wild type as was quantified by both techniques . Images of confocal microscopy of maximum projection of cells stained with Hoechst and H4 antibody were analyzed to obtain the ratio of H4/DNA content. (Figure 6)

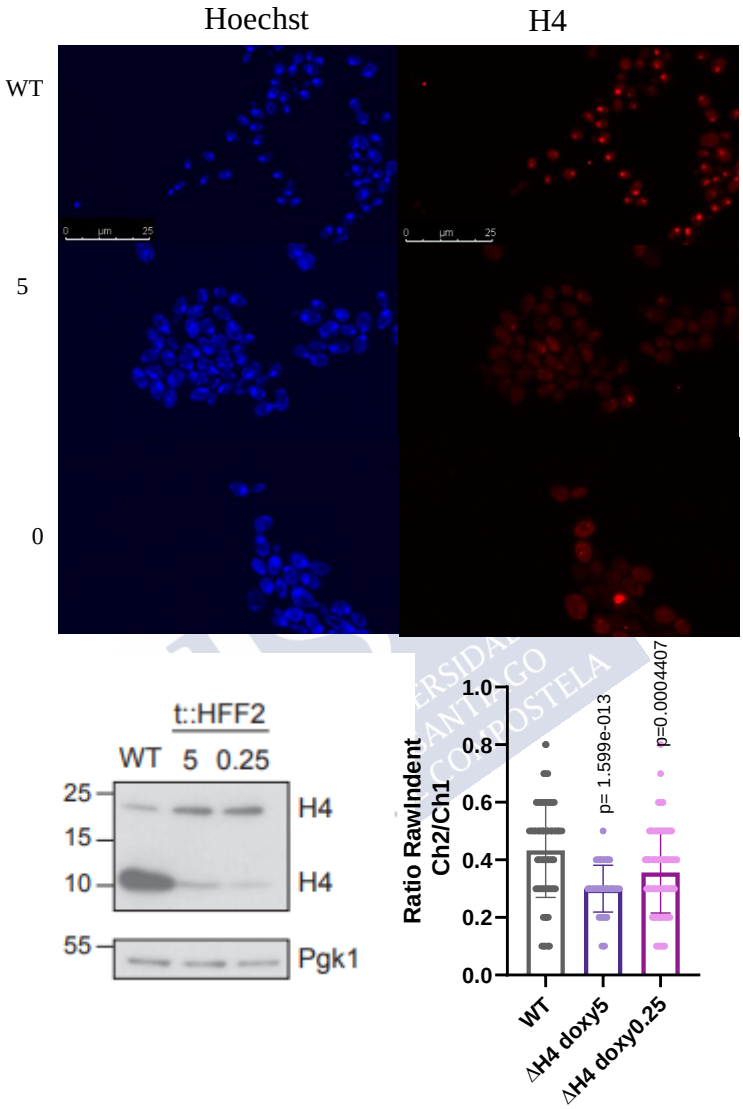


Figure 6. UP: Images of WT and ΔH4 cells treated with 5 μg/mL or 0.25 μg/mL of doxycicline. Left images show Hoechst dye and right, H4 antibody. Down left: WB of reduced expression of H4 in mutant strains (ΔH4) compared to WT. Down right: Ratio between H4 raw integrated density/Hoechst raw integrated density. Both 0.25 and 5 doxycicline induced cells of ΔH4 strain are lower than WT. ($p<0.05$). Graph shows individual points, mean and SD. Welch ANOVA test was performed in N>100 cells.

5.2.2 **DNA-drug binding in H4 partially depleted yeast model**

5.2.2.1 CDDP binding in H4 partially depleted yeast model

CDDP binded to DNA was quantified by ICP-MS of purified DNA samples. Cells were synchronized with nocodazole (antimitotic agent) and then incubated with CDDP 0.5 mM for 4 h. Cells with partially depleted H4 expression show increased levels of Pt/P compared to ratio of WT, but variability was high and biological replicates are needed. In this preliminary data we could see a change in average as big as almost a 4 folds increased ratio in average of Pt content in DNA. (Figure 7)

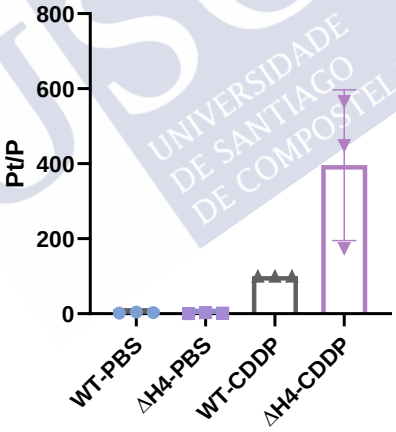


Figure 7. Pt content in DNA of WT and ΔH4 strains incubated with PBS or CDDP. The data is standardized by calculation of percentage compared to WT cells treated with CDDP 0.5 mM (100 %) and show mean±SD of three technical replicates. Cells with reduced H4 show an increase of almost 4 folds in average of bound Pt in DNA after a 4 hour treatment compared to WT. However, biological replicates must be performed.

5.2.2.2 Doxorubicin binding in H4 partially depleted yeast model

DOX binding was quantified directly by quantification of fluorescence by flow cytometry (DOX content in cells). An increase in DOX inside cells was observed in ΔH4 cells (in both doxycycline doses) compared to WT (WT). Results could be seen in figure 6. DOX results are in line with those observed in CDDP treatment in H4 depleted cells. However, since the measure is more indirect in this case, interpretation is more complicated. First, DOX content is only measured in cells but we could not differentiate the amount bound to DNA, RNA, etc. Second, cell cycle results that will be commented next, show an increase content of SYTOX green in H4 partially depleted cells, that could be interpreted both as a increase of DNA content (then DOX increase could be sobreestimated by the augment in DNA content in muntant cells) or by an increase in SYTOX intercalated in the same amount of DNA.

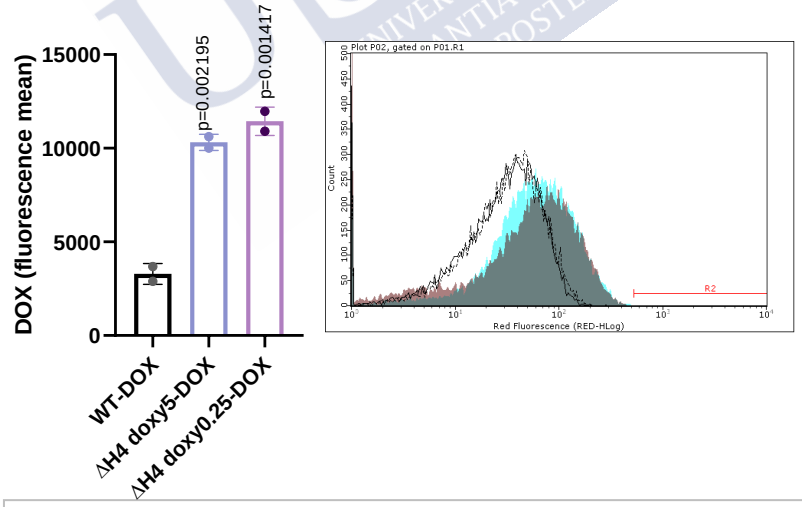


Figure 6. Left: DOX content in WT and ΔH4 strains with 5 or 0.25 μg/mL of doxycycline. Mean ±SD of averaged replicates. P values of comparison of means by ANOVA followed by multiple comparisons tested by Dunnet test. Right: Flow cytometry profiles. WT (transparent) ΔH4 (blue 0.25 and grey 5).

5.2.3 **Cell cycle of H4 partially depleted yeast model**

Cell cycle was analyzed to study the state of cells with the mutation since H4 depletion has a logical impact in cell cycle progression. Furthermore, to verify the effect of nocodazole arrest performed before DNA binding drugs treatments. Cell cycle profiles of WT and ΔH4 cells before nocodazole, right after nocodazole arrest and after 4 h of PBS treatment show the efficacy and maintaiment of arrest during treatment. However, the displacement in profiles of mutant strains that show high fluorescence of SYTOX green could not be determined. It could be a result of an increase in DNA content or a different entrance of SYTOX green (since mutant cells presented different size, morphology and reduced chromatin compaction). (Figure 8)

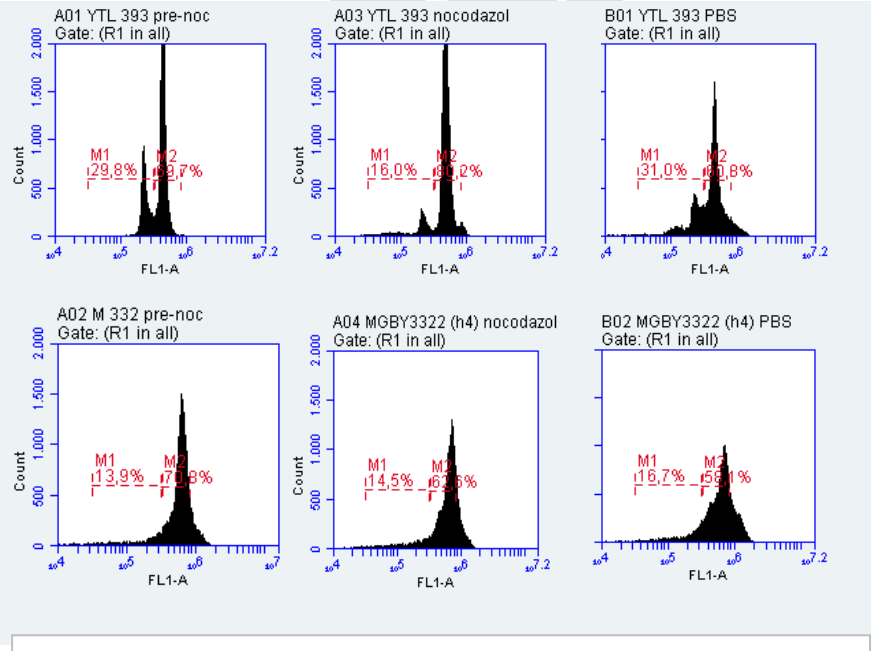


Figure 8. Cell cycle profiles of WT (YTL 593) (UP) and ΔH4 (MGBY3322) (DOWN) strains with 5 μg/mL of doxycicline. From left to right during experiment phases: before nocodazole arrest, at nocodazole arrest and after treatment with PBS. There is a displacement of profiles in H4 depleted cells (MGBY3322) that could be interpreted as an augment in DNA content over 4N or a different bound ratio of SYTOX/DNA.

5.3 SILENCING MODELS

After the study of chromatin compaction effect in yeast and ESCs genetic models, the next step was testing a human cell model that could be translated to the clinic. This was done by silencing proteins implicated in nucleosome formation, CAF-1 p60 subunit, and histone expression, CASP8AP2. Chromatin-Assembly Factor 1 (CAF-1) is a three-subunit histone chaperone (with subunits p150, p60 and p48) that specifically deposit newly synthesized H3-H4 histones onto replicating DNA during S phase, and CASP8AP2 is a transcriptional regulator of canonical histone genes.

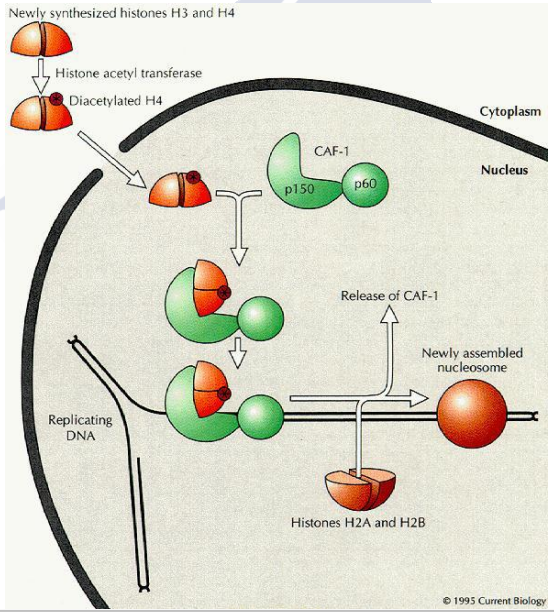


Figure 9. Scheme of CAF-1 role on nucleosome assembly during DNA replication. 1. Krude T. Chromatin: Nucleosome assembly during DNA replication. Current Biology. 1995;5(11):1232-4. DOI: https://doi.org/10.1016/S0960-9822(95)00245-4. Copyright© 1995 Elsevier Science Ltd. Published by Elsevier Inc. Use authorized by Elsevier, License number 4684021248380

In order to reduce its chromatin compaction, adenocarcinoma human cell line A549 was transfected with siRNA that target CASP8AP2 mRNA, chaf1b mRNA (which codifies p60 subunit of CAF-1), non-targeting siRNA (negative control) or GAPDH siRNA (silencing positive control).

5.3.1 **Expression after silencing**

Silencing of both proteins was performed with Darmacon siRNAs and DarmaFECT tranfection medium. 48 h after silencing, RNA was extracted to perform RT-qPCR to test silencing efficacy. The $\Delta\Delta C_T 2$ is increased in all silenced cells for its probe compared with non targeting-siRNA transfected cells and untreated cells. Increased $\Delta\Delta C_T 2$ indicates a reduced amount of mRNA in silenced cells. Thus, we could consider that silencing was successful. (Figure 10)

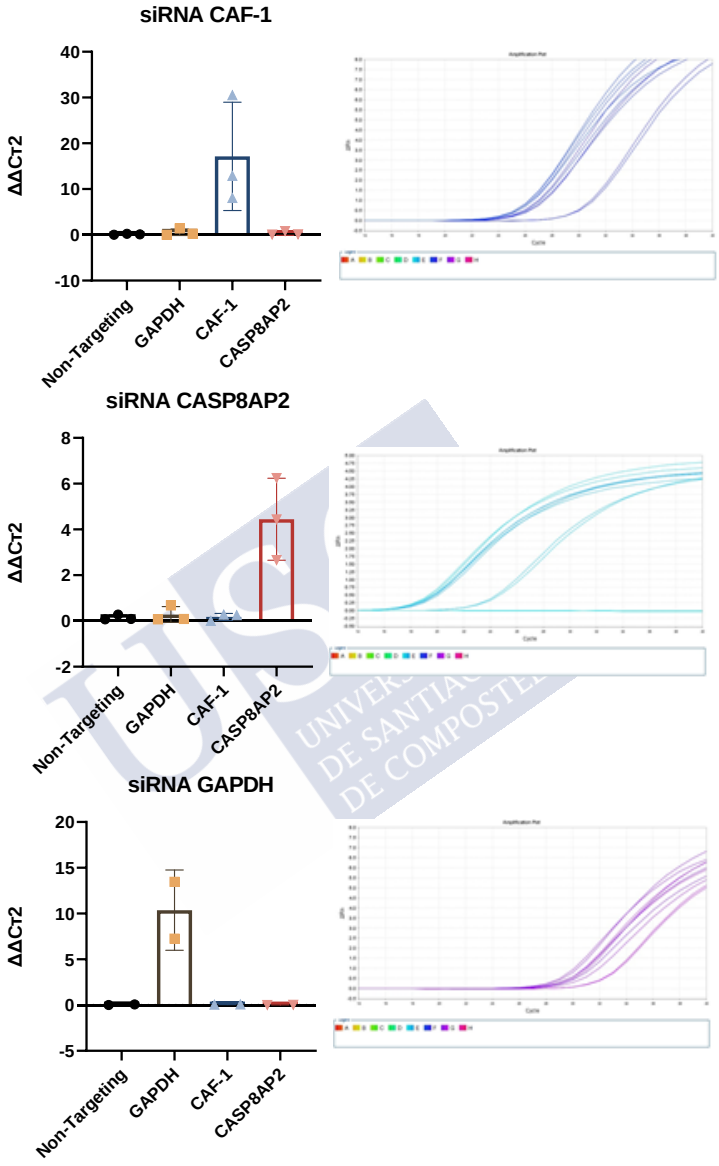


Figure 10.RTq-PCR results of CAF-1, CASP8AP2, and GAPDH silencing. In the left graphs we could see ΔΔCт2 of untreated cells, cells transfected with non-targeting si-RNA, GAPDH silenced cells, CAF-1 silenced cells and CASP8AP2silenced cells. Mean of 3 replicates in CAF-1 and CASP8AP2. In right amplification plots for each gen.

5.3.2 **DNA-drug binding in silencing models**

Only DOX treatment was studied in these models and DOX-DNA interaction was directly measured by flow cytometry. In CAF-1 silencing cells, 48 h after transfection, DOX was added for 4 h. DOX in CAF-1 silenced cells was almost 50 % higher than in cells transfected with non-targeting siRNA. (Figure 11)

In CASP8AP2 silencing model, DOX was added 72 h after transfection for 4 or 24 h. Figure 8 displays results of said experiments. DOX binding was only augmented in 24 h incubation and only a 10 % increase was observed. (Figure 11)

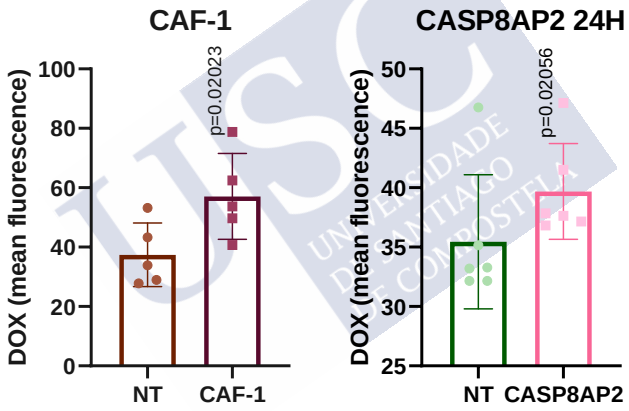


Figure 11.A549 cell uptake of DOX in CAF-1(left) an CASP8AP2 (right) silenced cells versus non-targeting transfected cells. Graph shows results of mean+-SD of 5 and 6 experiments, respectively. P values of t student mean comparison and Mann-Whitney median.

These models based on the silencing of histone expression and deposition related proteins present several limitations. First, silencing produces a reduction in viability and cell proliferation, as these proteins play important roles in the cell cycle, inducing cell cycle phases elongation or even apoptosis in some cells. Second, silencing still presents several limitations for clinic application.

5.4 iHDACs

Finally, we study the effect of iHDACs in drug binding to DNA. This type of combination (of iHDACs and CDDP or DOX) has proved useful in cancer treatment. However, since these drugs have a wide range of effect on cells, other explanations were given. Here, we propose that potentiation of CDDP and DOX efficacy resultant of their combination with iHDAC could be at least partially explained by the ability of iHDAC to reduce chromatin compaction and subsequently increase drug binding to DNA.

HDACs promotes the hydrolysis of N-acetyl lysine residues in histones, which increases histone affinity for DNA as it augments their positive charge. Inhibiting such enzymes will boost histone acetylation, changing histone charge and therefore promoting their release form the nucleosome.

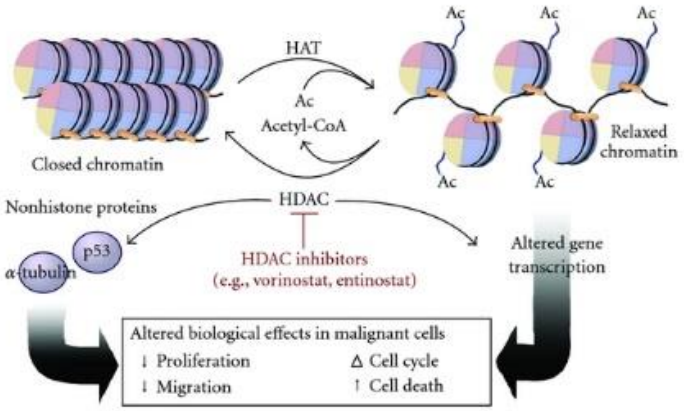


Figure 12. Schematic representation of acetylation effect on chromatin compaction and how iHDACs relate to higher levels of acetylation of histones lead to relaxed chromatin. Annabelle L. Rodd, Katherine Ververis, and Tom C. Karagiannis, "Current and Emerging Therapeutics for Cutaneous T-Cell Lymphoma: Histone Deacetylase Inhibitors," Lymphoma, vol. 2012, Article ID 290685, 10 pages, 2012. https://doi.org/10.1155/2012/290685.

We used three iHDACs: TSA, SAHA and VPA. TSA and SAHA act on clasess I y II HDACs. Then, TSA also acts on some class III and SAHA on class IV. VPA is weaker and has effect in class I and IIa HDACs only. We used a different factor that affects PMTs, procaine, a DNA metylation inhibitor. As in previous models, we used adenocarcinoma A549 cell line.

This model has some advantages compared to previous ones, as it is cheap, easy and has a direct impact in clinic. Since acetylation can occur in any moment since histone formation, their effect is not limited to cells with replicating DNA, as in the case of silencing models.

5.4.1 **Cell viability in iHDAC combination model**

In order to test the combination of iHDACs and DNA binding drugs, we first studied cell viability and proliferation. A549 cell line is sensitive to both CDDP and DOX alone, as shown in figure 9.

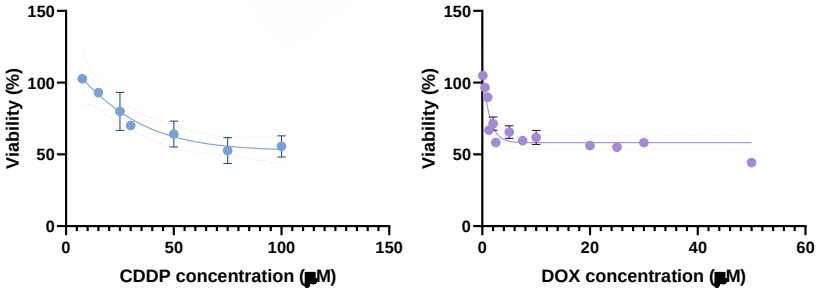


Figure 13. Cell viability curves A549 cells treated with CDDP and DOX Graph shows results of mean+-SD and 95 % CI.

A dose-response combination experiment was then performed. A549 were treated with: TSA (25 nM, 50nM or 75 nM), SAHA (0.75 μM, 1.25 μM or 2.5 μM), VPA (100 μM, 200 μM, 400 μM or 600 μM), procaine hydrochloride (25 μM, 50 μM, 100 μM) or CDDP (50 μM) alone or the combination of iHDACs in all this concentrations with CDDP. Data was normalized by results of treatment of CDDP alone. In figure 14, the graph shows % of cell viability of cells treatead with every concentration of iHDAC, alone or combined, standardized by cell viability of cells treated with CDDP alone. Thus, values below 100 show decreased cell viability of cells. All tested TSA concentrations and SAHA 1.25 μM and 2.5 μM showed reduced cell viability.

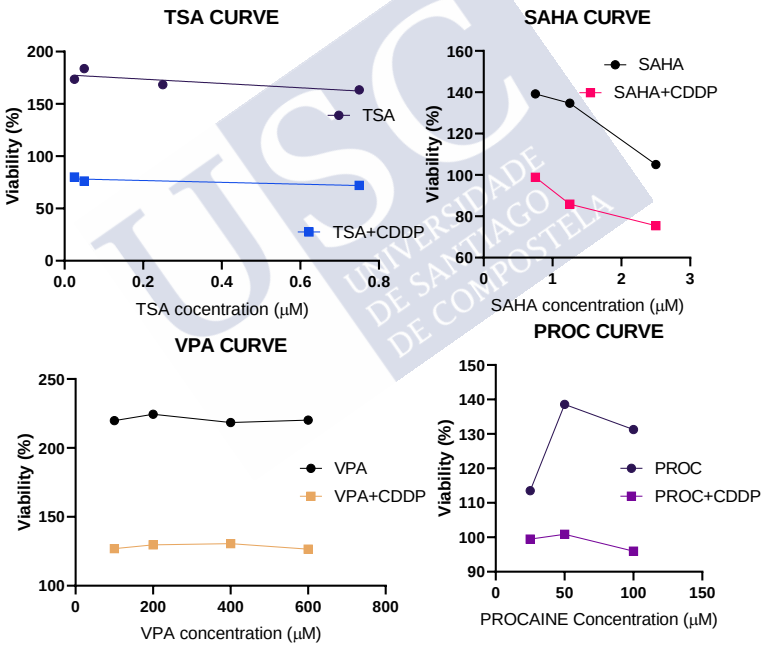


Figure 14. Dose-response curves of different concentrations of iHDACs alone or combined with CDDP 50 μM. Data is normalized by percent calculation related with CDDP treatment alone (100%).

After choosing the correct dose for each treatment, we selected subletal doses (alone) with an effect on cell viability in combination. The combination of TSA, SAHA, VPA and procaine hydrochloride was tested in two settings: short pretreatment of iHDAC (or procaine hydrochloride), followed by treatment with CDDP and cotreatment of CDDP and iHDACs.

When cells were treated for 4 h with iHDACs and then treated with CDDP for 24 h, we observed a reduction in cell viability in both TSA and SAHA combinations. VPA and procaine hydrochloride did not affect cell viability (Figure 15).

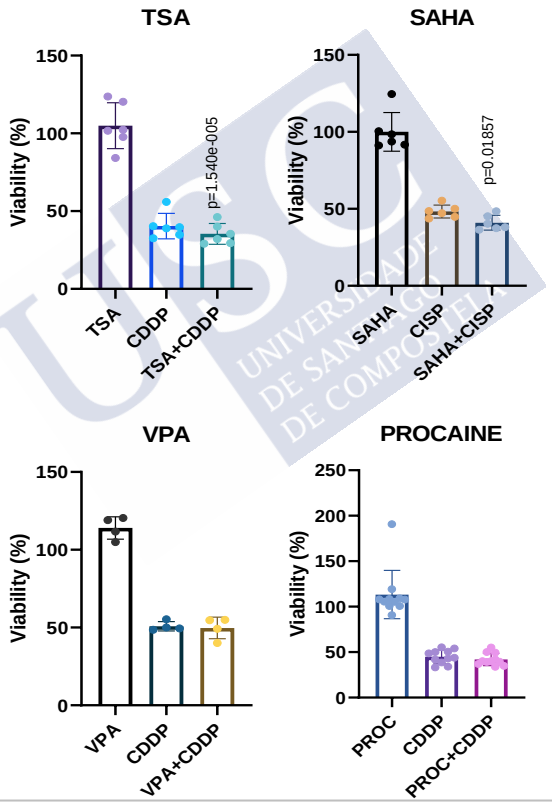


Figure 15. Cell viability of A549 cells pretreated with iHDACs and then treated with CDDP. Percentage of cells after treatment with CDDP, iHDACs or combnation: TSA 33 nM (A), SAHA 1.25 μM (B), VPA 200 μM (C) and with methylase inhibitor procaine hydrochloride 50 μM. The data is standardized by calculation of percentage of cells compared to untreated cells (100 %) and shows mean±SD of at leat 3 replicates . Unpaired t- student test was performed to compare CDDP alone or combination with SAHA, VPA and PROC and welch test to compare TSA combination.

A549 cells were also treated for 24 h with iHDACs (TSA 50 nM, SAHA 1.25 μM, VPA 200 μM and PROC 50 μM), CDDP(50 μM) or both. Again, only TSA and SAHA combinations with CDDP result in reduced cell viability compared to CDDP treatment alone. In figure 16 graphs show the results as percentage of cell viability compared to untreated cells.

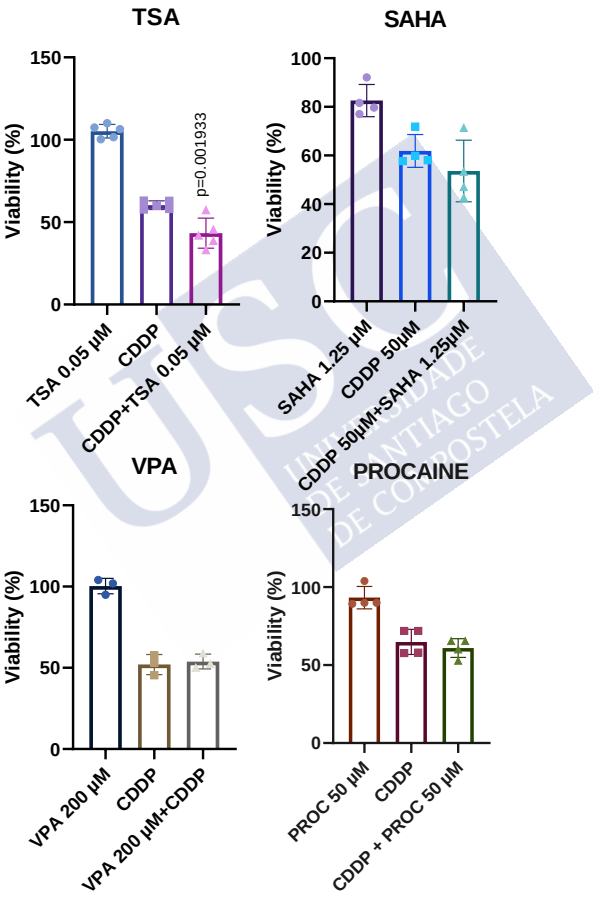


Figure 16. Cell viability of A549 cells treated with CDDP, iHDACs or combination (cotreatment). TSA 50 nM (A), SAHA 1.25 μM (B), VPA 200 μM (C) and with methylase inhibitor procaine hydrochloride 50 μM (D). The data is standardized by calculation of percentage of cells compared to cells treated with CDDP 50 μM alone (100 %) and shows mean±SDt student test was performed to compare means. (N>3).

5.4.2 **Cell cycle**

Additionally, DNA content of cells treated with TSA was quantified to observe the effect treatment has in cell cycle. A549 cells treated with 0, 50 or 100 nM of TSA and then dyed with PI. PI content was measured by flow citometry. In figure 17, we could see a graphic representation of cell cycle estimation based on the DNA content in cells untreated or treated with TSA (50 or 100 nM) for 24 h. A slight change can be seen in cell cycle distribution of cells treated with TSA 300 nM. G1 subpopulation is increased . This is interesting because, contrary to what happened in the yeast model, it means that cells with less DNA content have a higher content of DOX. DOX is already increased significally but binding may be underestimated.

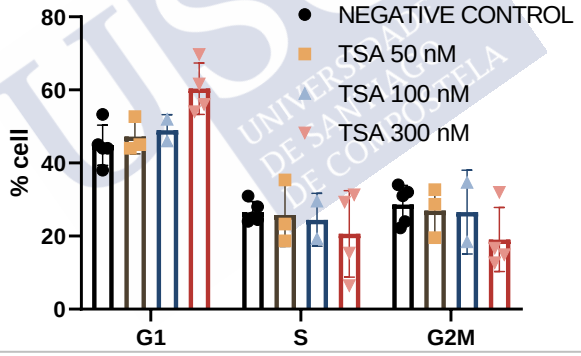


Figure 17. Cell cycle distribution of cells treated with 0, 50, 100 or 300 nM of TSA for 24 h. Mean ±SD of percentage of cells in each cell cycle phase.

5.4.3 **DNA-drug binding in iHDAC treated cells**

5.4.3.1 CDDP binding in iHDAC treated cells

CDDP binding to DNA was measured by ICP-MS quantification of elements Pt and P in DNA samples purified from cells: untreated, treated with iHDACs (TSA 300 nM, SAHA 2 μM and 4 μM and VPA 200 mM), CDDP (50 μM) or combination of iHDACs and CDDP. Both TSA and SAHA treated cells have higher Pt/P ratios than control cells. VPA ratios were similar to control ones. Therefore, TSA and SAHA combination produces an increased CDDP cytotoxic effect and DNA binding. While VPA does none.

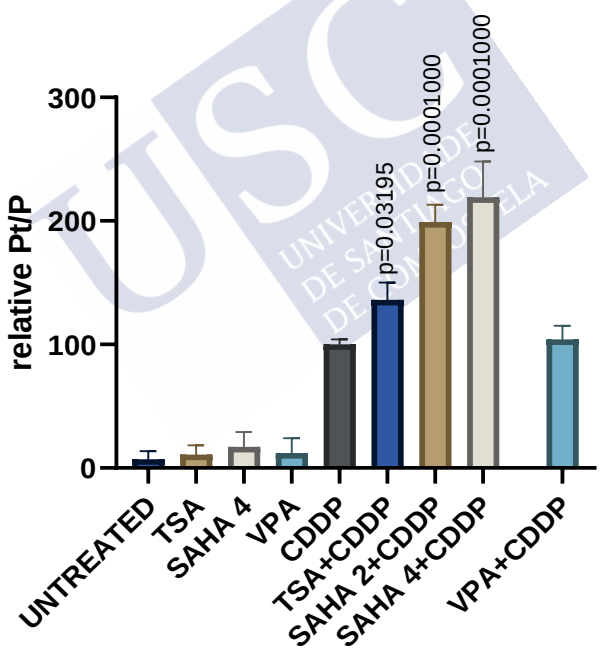


Figure 18. Pt content in DNA of A549 cells treated with CDDP, iHDACs or combination .TSA300nM, SAHA 2 μM or 4 μM or VPA 200mM.The data is standardized by calculation of percentage compared to cells treated with CDDP 50 μM alone (100 %) and shows mean±SD. One-way ANOVA and Dunnet were used to compare means of combinations vs CDDP alone.

5.4.3.2 Doxorubicin binding in iHDACs treated cells

To confirm the results found for CDDP we used DOX. DOX intercalation in DNA was measured in two directs ways using its fluorescence. A549 cells were pretreated with iHDACs for 24 h and then incubated with DOX for 4 h. After that, fluorescence was quantified by flow cytometry or confocal microscopy. Cells treated with TSA and SAHA showed DOX levels increased by 100% compared to those with DOX alone or with VPA and DOX. In figure 19A graphs show fluorescence mean of cells treated with each iHDAC and incubated with DOX and control just incubated with DOX. In figure 19B an example of flow cytometry profiles could be seen for each treatment.

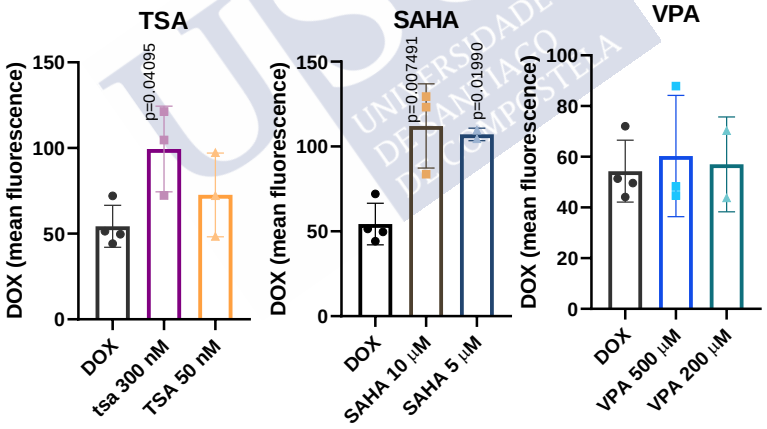
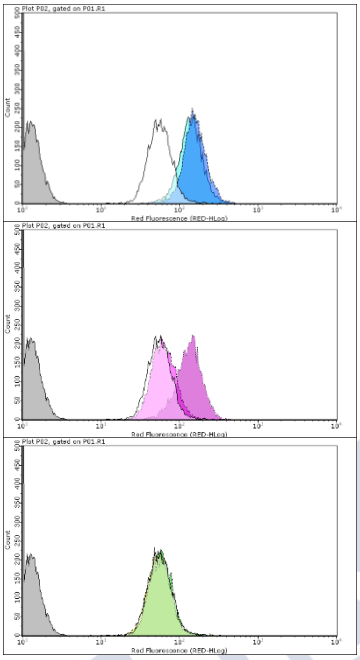


Figure 19A. Intracellular doxorubicin uptake measurement in A549. Mean fluorescence of A549 cells treated with DOX, iHDACs, combination or untreated. iHDACs were used in various concentrations: TSA 50 and 300 nM, SAHA 5 and 10 μM; VPA 200, 500 μM .mean±SD. Comparison of means was performed by one-way ANOVA and multiple comparisons with Dunnet test.



Negative Control	
Doxorubicin (DOX)	
SAHA 5 µM + DOX	
SAHA 10 µM + DOX	
TSA 50 nM + DOX	
TSA 300 nM + DOX	
VPA 200 µM + DOX	
VPA 500 µM + DOX	

Figure 19 B. Flow cytometric profile of A549 cells incubated with DOX (7.5 µM) for 4 h or pretreated for 24 h with SAHA 5 µM or 10 µM (D), TSA 50 nM or 300 nM (E) and VPA 200 µM or 500 µM (F) and then incubated with DOX for 4 h. Displacement of profiles are evident for both SAHA treatmets and TSA.

DOX localization in nucleus was confirmed through fluorescence confocal microscopy. Fluorescence was also quantified by image analysis. TSA treatment increased fluorescence in cell nucleus. Figure 16 contains a graph of fluorescence in cells treated with DOX (7.5 µM) alone or DOX and TSA (100 nM o 300 nM), and an example of images obtained.

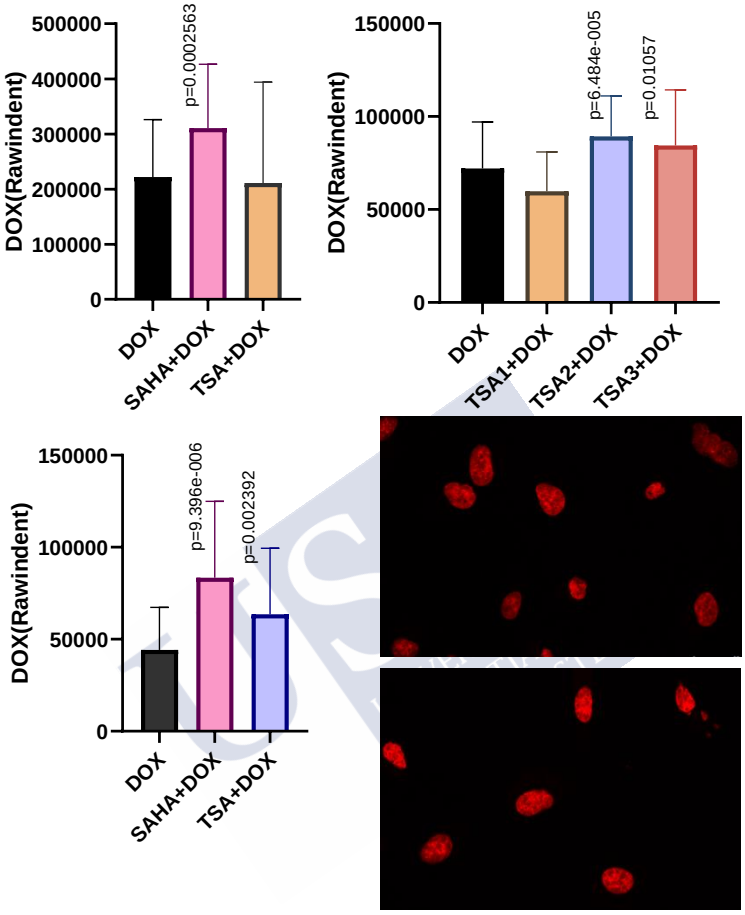


Figure 16. DOX content in nucleus of A549. TSA 100 and 300 nM (light and dark orange) and SAHA 2 μM increases DOX binding to DNA in A549 cell line. Mean of integrated density of fluorescence intensity of cells incubated with DOX alone or combined compared by ANOVA and Dunnet tests N>30 for all experiments. Confocal images of cells incubated with 7,5 μM of DOX for 4 h or with 300 μM TSA 24 h and then 7,5 μM of DOX for 4 h.

In summary, iHDACs SAHA and TSA that show potential increasing CDDP and DOX efficacy, also boost CDDP binding to DNA and DOX in nucleus. Conversely, VPA, that does not seem to affect CDDP or DOX levels, did not affect viability neither.

In all four models alteration in chromatin compaction could be related to an increase or a tendency of increased drug bound to DNA. Thus, even if models are not perfect, the chromatin compaction modification for cancer treatment is really promising.

6. DISCUSSION

Chromatin regulates gene expression, DNA replication and repair by limiting DNA accessibility, and thus acting as a barrier for the interaction of DNA with nuclear machinery (transcription factor, enzymes, etc.). [80] Drugs that binds to DNA found same barrier for their action. For example only 1% of CDDP inside the cell is found bound to DNA. [64] Previous works in this lab with Ag_3-AQCs, that intercalate into DNA and reduce chromatin compaction, improve drugs binding to DNA like oxaliplatin, CDDP, gemcitabine, DOX; and enhance their efficacy in tumor models. AQCs combination with CDDP resulted in an increment of 5 folds in CDDP bound to DNA and an improve of CDDP therapeutic index in tumor mouse models. [83] We explore whether reducing chromatin compaction by other mechanism can increase DNA accessibility for drugs. With this objective we choose two drugs: CDDP and DOX, that mainly acts directly by binding to DNA. CDDP mechanism of action by adduct formation with nucleotide bases is well described, besides CDDP can be quantified with precision by ICP-MS. [95,96,83.] DOX also form adducts with DNA and with its fluorescence became a perfect approach to make easy quantification in cells [97,98,83]. Despite similar mechanism of action, their chemistry, uptake, transporters, ect. are different which makes them good complementary drug models for our study. Our first approach was using an embryonic stem cells, in which a mutation introduced to provide resistance to an antibiotic results in reduced compaction of chromatin. After founding promising results, we wanted to test a more defined model. Yeast genetically modify to reduce histone H4 expression was our second confirmation that reduced chromatin compaction can increase CDDP and DOX binding. Nevertheless, as we are trying to

improve anticancer drugs efficacy, our theory should be tested in a model not only in human tumor cells, but also a model that could be translated to clinic. First, based on yeast model, a direct knockdown of proteins related with nucleosome assembly that have shown effect in chromatin compaction was tested. Results were really limiting in this model, so a new approach, using drugs that can reduce chromatin compaction, are already in use in clinic and have wider time range effect during cell cycle was choosen: iHDACs treatment.

6.1 ESC MODEL

First, we used an embryonic stem cell form mice. ESC form cj7 parental line was selected as a model as a mutant obtained by the insertion of the gene for resistance to an antibiotic in Zmym2 that disrupts expression of the protein originates a change in chromatin compaction.Zmym2 encodes a protein whose function is not well know but it could be a transcription factor or part of a complex involved in histone deacetylation [99].This model has the advantage of presenting an intrinsic reduced chromatin compaction, thus avoiding effects of pharmacological treatment will have in terms of interpretation. As we expected, DNA of cells with reduced chromatin compaction bind more CDDP. Mutant cells also show higher DOX content than WT cells, both measured by flow cytometry and nuclear content by fluorescence confocal microscopy. The change in drug binding (20-30%) was small compared to AQCs effect, nevertheless these results are really promising as the mechanism behind chromatin compaction change is different which suggest that chromatin compaction reduction is the cause behind drug binding increment. This model was a first approach to human cells without pharmacological modification but, ESCs culture requirements are expensive, and because our objective is the study of chromatin effect on anticancer drugs in human cells, we decide to continue with different models in which conditions are better controlled.

6.2 H4 PARTIALLY DEPLETED YEAST MODEL

S. cerevisiae is an experimental system extensively used for studying cellular processes in other species, including humans. As a model present some advantages like easier and cheaper genetic manipulation (in haploid and diploid forms), high similarity with humans cells in many molecular, genetic, and biochemical features with including functional pathways implied in cell cycle, metabolism, apoptosis, protein folding and degradation, signaling pathways, etc.[100,101,102] Also, both drugs we choose for this work were previously used in yeast. [103-108].

The first model used to study chromatin compaction was based on a strain created by Prado & Aguilera. In this strain H4 expression was reduced, which results in a reduction in chromatin assembly. H3 and H4 deposition on DNA is the first step in nucleosome formation after DNA replication. H4 depletion results in total inhibition of growth and reduced levels of expression leaded to important changes in cell biology of mutant cells like delaying growth due to extended S and G2/M phases, accumulation as budded cells, heterogeneous increase in cell size, less sensitivity to alpha-factor, genetic instability, etc. [85]

In our model, we compared two strains, the H4 partially depleted strain t::HHF2 and a strain with same background and transfected with the same plasmid that contains tet regulated expression in the other one, but without insert: *BY4741*.

CDDP binded in DNA was quantified by ICP-MS of purified DNA samples. Since mutant strain shows elongated S and G2/M phases and accumulation of cells in G2/M compared with WT [85] nocodazole (an antimitotic agent) was used to synchronize cultures, in order to have most homogeneous as possible setting for the experiment. After synchronization with nocodazole cells were treated with CDDP 0.5 mM for 4 h. The reduction in chromatin compaction in the strain depleted H4 expression show increased levels CDDP bound to DNA of 4 folds in average compared to BY474 (WT). An increase of 3 folds in DOX inside cells was observed in mutant cells compared to WT. DOX binding was

quantified directly by flow cytometry (DOX content in cells). DOX content increased in mutant as what happened with CDDP treatment. DOX content is only measure in cells but we could not differentiate the amount bound to DNA, RNA, etc. CDDP is described for example to bind more to RNA (especially ribosomal) than to DNA in yeast [109]. In CDDP experiments DNA is isolated and we quantify the amount of Pt/DNA what gives us a more reliable meassure. Second, cell cycle results show an increased content of sytox green (the DNA dye) in H4 partially depleted cells. This augment of sytox could be interpreted both as an increase of DNA content (which could interfere in DOX increase interpretation, as part of DOX increment could be a result of DNA augment) or by an increase in sytox binding in cells with the same amount of DNA. H4 partial depletion leads to genetic instability and mitotic aberrations so a change in DNA content can not be discarded. However, the change in chromatin estructure, size of cells (mutants are bigger) and shape (less round) can also lead to a change in sytox uptake and binding to DNA resulting in changes in flow cytometry fluorescence profiles not related with DNA content [110].Likewise, Prado and Aguilera did not describe a change in DNA content when they describe the strain in cell cycle results. [85] CDDP results are more robust, in terms of design but replicates are needed.

Despite the existence of many similarities there are also many differences between yeast and humans. For example, de novo synthesized histones are essential for cell cycle progression in human cells and not in yeast, that can complete one round of replication without new histone production. The same happens with CAF-1 necessary in human cells and whose delection have mild effect in yeast (which seem to growth normal after deletion of CAF-1 genes) that are thought to have an alternative pathway for nucleosome assembly. [86,88,111] Therefore after testing our approach in yeast we wanted to use a model with higher similarities to human cells. And also in models that can be translated to cancer treatment.

6.3 SILENCING MODELS

Our first approach to an orientated clinical model was silencing of two proteins related with histone expression and deposition in DNA, CASP8AP2 and CAF-1, in A549 human lung adenocarcinoma cell line. CASP8AP2 is a transcriptional regulator of histone genes and Chromatin-Assembly Factor 1(CAF-1) is a three-subunit histone chaperone (with subunits p150, p60 and p48) that specifically deposit newly synthesized H3-H4 histones onto replicating DNA during S phase. [86,87]

DNA replication is closely matched with chromatin assembly. For chromatin assembly both parental recycled and newly synthesized histones are required. In human cells loss of histone expression or of nucleosome assembly factors like CAF-1 or ASF-1 leads to cell cycle arrest or other cell cycle alterations. CASP8AP2 orchestrates expression of replication dependent histones (canonical histones) and participates in histone mRNA initial processing. Sokolova et al. results shows that CASP8AP2 depletion in U2OS (osteosarcoma cell line) and hTERT-RPE1 (hTERT- immortalized normal retinal pigment epithelial cell line) cells results in reduction of H3 levels in both lines. In different cell lines several studies show how CASP8AP2 silencing effectively results in H3 reduced levels. However, the subsequent effects were different: elongated S phase, arrest in S-phase, apoptosis, etc.[121]

CAF-1 depletion resulted in decreased nucleosome assembly in various studies, but other consequences were different in different vertebrate cell lines. Similary to the effect in CASP8AP2, depletion of CAF-1 results in reduction of nucleosome assembly, but other effects, like elongated S-phase, apoptosis or cell death was observed. [111-113]

Despite commented differences, both proteins depletion show stable effect in nucleosome formation among different cell lines for which they were selected in this work. Adenocarcinoma human cell line A549 was transfected with siRNA that target CASP8AP2 mRNA, chaf1b mRNA (that codifies p60 subunit of CAF-1) in order to reduce its chromatin compaction, or and siRNA without a

target in cells (as negative control). Results obtained in these models agrees with previous approaches as DOX content in both cells with reduced chromatin compaction due to CASP8AP2 and CAF-1 p60 depleted cells was higher that in controls. This is the third model with different background in which changing nucleosome assembly correlates with an increase in drug bound to DNA.

This model presents some incovenients as well, in both cases silencing leads to a reduction in cell proliferation, which concordats with previously mentioned works, as these proteins play important roles in cell cycle, inducing cell cycle phases elongation or even apoptosis in some cells. Because of that as time progresses after silencing, we will be selecting cells without silencing among population, that will be proliferating faster. Also, with transient silencing and toxic transfection medium, this model was very limitated in terms of type of experiments that could be performed. Additionally, the increase in drug binding around 20% in CAF-1 silencing model and 5% in CASP8AP2, was small compared to the one found in AQC that could actually have clinical relevance. [72] a different model was consequently selected. Treatment with iHDACs., that have been proved able to reduce chromatin compaction. [93]

6.4 iHDACs

The last model to study the effect of chromatin in drug binding to DNA was a wide studied type of drugs cancer therapy and several other treatments: iHDACs. [114-117] HDACs promotes the hydrolysis of N-acetyl lysine residues in histones which increases histone affinity for DNA as augments their positive charge. Inhibiting HDACs will increase histone acetylation and causing their release form nucleosome. We used three iHDACs: TSA, SAHA and VPA. TSA and SAHA are hydroxamic acid derivatives, with wide range of HDACs targets. On the other hand, VPA is a short chain fatty acid with weaker activity against a narrower range of targets. TSA was selected because its effect on global chromatin

compaction has been proved. [93] Our results concord well with this as SAHA and TSA show same tendency of increasing CDDP and DOX binding to DNA as well as cell viability; while VPA treatment has no effect in any of those.

This kind of combination (of iHDACs and CDDP or DOX) has proved to be useful in cancer treatment in many occasions. [3, 114,115] However, since these drugs have many effect on cells, other explanations were given. Potentiation of DNA binding drugs by iHDACs was associated with changes in expression patterns, in apoptosis pathway, in redox state, etc. [114,115,117-120] Here, we propose that potentiation of CDDP and DOX efficacy resultant of their combination with iHDAC could be at least partially explained by the ability of iHDAC to reduce chromatin compaction and subsequently increase DNA accessibility to drug binding.

This model has some advantages compared to previous ones, is cheap, easy and has a direct impact in clinic. Since acetylation can occur in any moment since histone formation, their effect is not as much limited by cell cycle like silencing models. Although canonical histones are expressed during replication and excess of histones is control to avoid toxic effect [78], other variants are expressed in different moments [77] Moreover, while TSA is used as a laboratory drug due to its high toxicity in vivo, SAHA is already approved for clinic use. [6] There are also other iHDACs of hidroxamic acid group that are approved for clinical use like Belinostat and Pabinostat and more others in clinical trial. iHDACs of other groups with different HDACs targets could be also potentially studied.

In summaryinall four models of chromatin compaction modification could be related with an increase in drug binding to DNA.

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7. CONCLUSIONS

1. Chromatin compaction reduction by genetic manipulation of histone levels in yeast improves drug binding to DNA.

2. Reduced chromatin compaction, produced by the mutation in the zmym2 gene, is related with increased anticancer drugs binding to DNA in murine embryonic stem cells (ESCs)of CJ7 parental line.

3. In human lung adenocarcinoma A549 cell line silencing of CAF-1 and CASP8AP2, that reduce nucleosome formation, increases albeit at low levels DOX binding to DNA.

4. In human lung adenocarcinoma A549 cell line, the use of iHDACs TSA and SAHA, that decrease chromatin compaction, increase the binding of anticancer drugs to DNA and, thus, augments their therapeutic efficacy.

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